STUDIES ON THE CYTOTOXIC EFFECTS AND MECHANISM OF ACTION OF 1-PHENYL-3-PHENYLAMINO-4-(P-TOLUENESULFINYL)-TRANS-1,5-HEXADIENE

by

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Literature Review

INTRODUCTION

In 1937, Congress created the National Cancer Institute, whose mission has been and continues to be the investigation of the nature of cancer and its cause and prevention, diagnosis and cure (114). Within the NCI, the Division of Cancer Treatment (DCT) is responsible for monitoring the national effort to control cancer through surgery, radio therapy, immunotherapy, and combined modality therapy (114), in addition to the development and evaluation of new drugs. It is this last aspect in which we are most interested.

Research conducted during World War II, particularly on antibiotics, led to the development of organized research programs in cancer chemotherapy. Mass screening programs of crude fermentation products by the pharmaceutical industry resulted in the isolation of several compounds which were later shown to have antitumor activity (114).

The Cancer Chemotherapy National Service Center (CCNSC) was established in 1955 (78), and after several name changes, was merged with the DCT in 1973 (114). A recommendation was made in late 1957 that the CCNSC should begin a broad screening program of plants. It was suggested that the Compositae, the Euphorbiaceae, the Labiatae, the Leguminosae, the Lilliaceae, and the Umbelliferae plant families be screened first (78). Contracts were let for plant collection, extraction, and screening of the extracts. The level of screening of extracts throughout the years has varied according to the budget of the NCI (78). In addition to plant extracts, synthetic products,

marine animal extracts, and fermentation broths have also been screened for activity (78). As of 1980, approximately 178,802 microbial cultures have been isolated, and 103,272 plants have been extracted. In addition, 13,751 extracts from marine products have been screened, and 0.7% have shown confirmed in vivo activity (27).

A network planning and control technique, the "convergence technique", has been applied to the natural products screening program (115). This technique employs decision points throughout the new drug development process which allow only the most promising compounds at each step to continue through the screening process, thus allowing conservation of resources. A prescreen is used to test all compounds. The activity level is set low in acknowledgment of the fact that no one tumor cell system can predict the activity of all active compounds against different tumors (115). The prescreen used over the years has changed due to back-study correlations between active compounds and activity against the prescreen. The current prescreen in use is P388 murine macrophage leukemia/lymphoma (105). The P388 prescreen is effective in predicting activity. It is sensitive to most classes of clinically effective drugs but sufficiently discriminating (105). In addition, the P388 cell line has been shown to be exceedingly stable to changes over time (65).

There is a substantial fallout of active compounds after the prescreen. Only the most active compounds go on for further testing to the tumor panel screen. However, some promising compounds are eliminated due to the problem of obtaining sufficient quantities of the compound for testing. It is in this area that synthetic organic

chemistry is of value. If a relatively simple synthesis with high yields can be developed, then promising compounds need not be eliminated from screening. Also,the total synthesis of known active compounds would eliminate the need to gather and extract the compounds from plants or other natural sources, thus saving time and expense (27).

Synthetic organic chemistry is also of value in preparing analogs of known anticancer drugs. There are many reasons why analogs are of importance. Primarily, it is hoped that analogs which show increased activity and decreased toxicity in comparison with the parent compound will be developed (112, 27, 13).

The development of analogs is important to different people for different reasons. For the organic chemist, analogs are useful: for structure-activity determinations which lead to the rational synthesis of new and improved drugs for cancer treatment (27); for developing drugs with improved solubility (13); to separate the components of the spectrum of action of a drug, such as the toxicity and the antineoplastic activity, into separate entities (58); to develop drugs with differences in tissue specificity (58).

For the cancer research scientist, analogs are useful for determining the mechanisms of action of compounds, which leads to greater selectivity in choosing drugs for particular diseases (27). Analogs may: affect a different phase of the cell cycle than the parent compound (13); exhibit more selective tumor cell killing (13); show greater retention of activity at various stages of the tumor cycle(13); or, exhibit more extensive action against nondividing

tumor G_o cells (13). In addition, analogs may: exhibit increased entry into tumor cells and/or increased binding to active sites (13); overcome or delay tumor cell resistance (13); demonstrate therapeutic synergism when employed in combination with a parent drug or structurally unrelated compound (13); show greater effectiveness in preventing metastasis or in the treatment of metastatic tumors (13); or, show greater activity against oncogenic viruses, viral transformations, or viral-induced tumors (13).

For the pharmacologist/clinical oncologist, analogs are desired for the following reasons: a broader spectrum of effectiveness against experimental tumors (13); diminished limiting or undesirable acute, chronic, or delayed host toxicity such as myelosuppression,or gastrointestinal, cardiac, or lung toxicity (13); improved stability to metabolic destruction or excretion (27); improved duration of activity and selectivity for a target site (27); greater maintenance of blood and tissue concentration levels (13); an increase or decrease in lipid solubility to cross the blood brain barrier (112); more satisfactory dosage form including greater solubility or greater stability for oral administration (13); greater effectiveness over a variety of dosage schedules, and more latitude in the choice of routes of administration (13).

ANTICANCER AGENTS

Interference with the synthesis or function of nucleic acids, or with the mitotic process itself is ultimately the mechanism by which most if not all agents kill cancer cells (70). There are several classes of anticancer agents, including: hormones; chemically reactive agents; antimetabolites; DNA complexors; mitotic inhibitors; and, other agents. As may be expected, some agents fit into more than one class, and not enough is known about some new agents to enable their placement into a class. These classes of anticancer agents will be discussed below. The sheer volume of material already written about many of these compounds precludes more than a cursory discussion.

HORMONES

In this section, not only will some of the hormones used in cancer treatment be considered, but also some of the hormone antagonists.

Unlike the sex hormones which act on a selected group of target tissues, glucocorticoids act on a wide variety of tissues and organs. Prednisone, a synthetic steroid, is not active when given orally until it has been converted in vivo to prednisolone (82). These drugs, like other glucocorticoids, are cytotoxic to lymphoid tissue, but not to the bone marrow. Prednisone has been useful in the treatment of acute lymphoblastic leukemia, and other diseases such as breast cancer, Hodgkin's disease, and multiple myeloma. Because the drugs affect many different types of cells, there are a large number of possible

side effects which may include ulcers, cardiovascular and renal hypertension, and immunosuppression resulting in infections (82). Because prednisone is not cytotoxic to the bone marrow, it has been used extensively in combination therapy with other drugs; rarely is prednisone used as a single anticancer agent (82).

Antiestrogen treatment has become an important strategy in the management of breast cancer. Approximately 30% of postmenopausal women with advanced breast cancer respond to endocrine therapy. In these patients, circulating androgens in the peripheral tissues are converted to estrogen by the estrogen synthetase enzyme complex, aromatase (38). By inhibiting this enzyme, tumors are deprived of estrogen. A new agent, 4- hydroxyandrostenedione, selectively inhibits aromatase, with fewer side effects than aminoglutethimide. This latter agent inhibits at an earlier step in steroid biosynthesis, thereby leading to depletion of corticosteroids, in addition to other side effects (38).

Tamoxifen is a nonsteroidal antiestrogen used in the treatment of breast cancer. Not only has tamoxifen shown tumor cell estrogen receptor blockade (39), but also the inhibition of proliferation of estrogen receptor-negative cancer cells (42). Research has indicated that tamoxifen inhibits Ca^{+2} influx and competes for Ca^{+2} channel antagonist binding sites (39). This effect may account for some of the side effects of tamoxifen, including vasomotor disturbances, nausea, vomiting, peripheral edema, and headache. These side effects may be related to the inhibition of Ca^{+2} flux in smooth muscle (39).

Another antiestrogen, nafoxidine, has been shown to have antitumor activity against an ascitic hepatoma. Nafoxidine competitively inhibited estradiol binding to the tumor cells (55).

CHEMICALLY REACTIVE COMPOUNDS

The chemically reactive compounds are mostly alkylating agents which act primarily by transferring alkyl groups to cellular constituents (56). This class includes the nitrogen mustards, aziridines, methane sulfonates, imidazoletriazenes, and the nitrosureas.

Cyclophosphamide is probably one of the best known nitrogen mustard compounds. The transport form of the drug, 4-hydroxycyclophosphamide(18), is activated by the hepatic cytochrome P450 (72), a mixed function oxidase system, to form phosphoramide and acrolein. Phosphoramide mustard is the alkylating/crosslinking moiety (18), and acrolein, a highly reactive compound, causes DNA single strand (ss) breaks and the depletion of glutathione (19).

Melphalan is a nitrogen mustard derivative of L- phenylalanine, and has been demonstrated to be transported into tumor cells by two amino acid carrier systems, the large amino acid carrier system and the sodium-dependent alanine-serine- cysteine transport system (40). Research results suggest that melphalan is able to be transported across the blood brain barrier via the large amino acid carrier system in a facilitated, concentration-dependent manner (40). Melphalan is a bifunctional alkylating agent capable of producing DNA intrastrand, and DNA- protein crosslinks (44). However, cell sensitivity to this

type of drug is determined not only by the number of crosslinks produced by the drug, but also by the cell's capacity to repair crosslinks and rate of repair (44). Melphalan activity is potentiated by the use of buthionine sulfoximine (BSO)(59). BSO is an agent that can selectively inhibit the rate limiting enzyme in glutathione (GSH) biosynthesis, thereby depleting tissues of GSH (41). Glutathione has been shown to have a protective role against cellular injury (59). This suggests that thiol modulation by BSO or other agents may be useful as an adjuvant in cancer chemotherapy.

The aziridines are nitrogen mustards which cyclize to aziridinium ions in vivo. Parent compounds include triethylene melamine (TEM), and Tris(1-aziridinyl)phosphorine oxide (TEPA)(70). In newer compounds, the aziridinyl groups are combined with other reactive groups, most often quinone structures. Aziridinyl groups are thought to react with DNA, after opening the ring, in the same way as other alkylating agents, thus leading to crosslinks (113).

The methane sulfonates are compounds containing a sulfonic acid ester of nitrogen mustard. Busulfan was used for chronic myeloid leukemia, but efforts to improve the drug were unsuccessful (70). More recently, haloethyl sulfonates have been tested. 2-Chloroethyl(methylsulfonyl) methanesulfonate crosslinks DNA after an initial alkylation at the O^6 position of guanine (34). Another compound, 1,5,2,4-dioxadithiepane-2,2,4,4- tetraoxide, may also react as a bifunctional alkylator. This cyclic compound, however, is thought to become a negatively charged intermediate after the first reaction, which may then react preferentially with positively charged

histones which surround the DNA helix. This results in DNA-protein crosslinks rather than DNA interstrand links (34).

The nitrosureas are both alkylating and carbamoylating agents. BCNU, 1,3-bis(2-chloroethyl)-1-nitrourea, and CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea have been most effective against Hodgkin's disease, non-Hodgkin's lymphoma, certain brain tumors, and malignant melanoma. However, bone marrow suppression is delayed, prolonged, and cumulative (70). The chloroethyl nitrosureas are unstable compounds that decompose to a variety of reactive species (35). Two types of alkylation reactions have been demonstrated: chloroethylations and hydroxyethylations. Chloroethylation of the guanine O^6 position is followed by a slow series of reactions leading ultimately to interstrand crosslinks. Side reactions such as the hydroxyethylations could produce unnecessary toxicity, do not contribute to and may interfere with antitumor activity (35). However, a majority of human tumor cells and normal cells are capable of repairing these crosslinks in a stoichiometric, hence depletable, manner (36). One current strategy used to overcome this is the pretreatment with the methylating agent N-methyl-N'-nitro-N-nitrosoguanidine. This causes sensitization to the effects of chloroethylating agents, but not to other alkylating agents that react primarily at the N^7 guanine position (36). However, this compound is highly carcinogenic. Alternatively, streptozotocin, an N-methyl-N-nitrosurea derivative of glucosamine, is used as a pretreatment (36). Another strategy is the post treatment with methylxanthines such as theobromine or caffeine (31). The postulated

mechanism of action is that the methylxanthines prevent delays in G2 of the cell cycle, thus increasing DNA damage by preventing sufficient time for repair.

Structure-activity studies have shown that the conjugation of a cytotoxic moiety to a specific site on a glucosamine molecule can alter the cytotoxic effects on normal and tumor tissue. The conjugation of nitrogen mustard to C-2 or C-6 of glucose resulted in P388 antitumor activity greater than that of nitrogen mustard alone, with a concomitant reduction in murine bone marrow toxicity (12). Two compounds with alkylnitrosureas conjugated to C-2 of glucose are streptozotocin and chlorozotocin. These also demonstrated reduced toxicity with the retention of antitumor activity (2). Conjugation of a mustard to C-6 of galactose, an isomer of glucose, resulted in a compound with slightly better activity and lower toxicity than the corresponding glucose compound (88).

ANTIMETABOLITES

Antimetabolites are compounds that interfere with the synthesis or function of nucleic acids. Included in this class are: inhibitors of dihydrofolic reductase; purine analogs; and pyrimidine analogs. Examples of these are discussed below.

Dihydrofolic Reductase Inhibitors

The best known inhibitors of dihydrofolic reductase are aminopterin and amethopterin (methotrexate). These 4-amino derivatives of folic acid are competitive inhibitors that prevent the

reduction of dihydrofolate to tetrahydrofolate. Tetrahydrofolate is then converted to a variety of coenzymes that are necessary for reactions involved in the synthesis of thymidylate, purines, methionine, and glycine. Thus, inhibition of dihydrofolic reductase can lead to inhibition of DNA, RNA, and protein synthesis (81). Methotrexate is useful in the treatment of several human cancers, but is not effective against many solid tumors, and is limited by toxicity to normal tissue. In addition, the development of resistance is a problem (61).

Trimetrexate, (2,4-diamino-5-methyl-6-[(3,4,5- trimethoxyanilino) methyl]quinazoline, is a potent inhibitor of dihydrofolic reductase (61, 6), although it does not resemble in structure either folic acid or dihydrofolic acid. Trimetrexate apparently differs from methotrexate in the mechanism of cell entry and intracellular metabolism (6). The drug shows activity both in vitro and in vivo: in vitro, it is active against methotrexate-resistant cells (61); in vivo, a broader range of activity than methotrexate against murine tumors is shown (6).

Purine Analogs

Of the purine analogs, 6-mercaptopurine and 6-thioguanine, have been widely used in chemotherapy. 6-Mercaptopurine is converted by the purine salvage enzyme Hypoxanthine guanosyl phosphoribosyl transferase (HGPRT) to 6-mercaptopurine-ribose- phosphate and 6-methylmercaptopurine ribonucleotide, both of which act on amidotransferase to inhibit de novo purine synthesis (81).

6-Mercaptopurine-ribose-phosphate also inhibits the conversion of inosinic acid to adenylic and guanylic acids. Also, 6-mercaptopurine is incorporated into nucleic acids as 6- thioguanine. Both mercaptopurine and thioguanine are used primarily to treat acute leukemias. The principal toxicity is myelosuppression, although liver toxicity also occurs (81).

3-Deazaguanine, another purine antimetabolite, has been shown to inhibit the enzymes involved in guanine nucleotide biosynthesis, including inosine monophosphate (IMP) dehydrogenase and aminoimidazolecarboxamide ribonucleotide transformylase (79). As with mercaptopurine, 3-deazaguanine must be activated via HGPRT to at least the ribonucleotide monophosphate for pharmacological action (79). The drug decreases L1210 cell viability and DNA and protein synthesis, but doesn't affect total RNA sythesis. In addition, GTP levels are lowered to as little as 10% of control values (79). 3-Deazaguanine is incorporated into nucleic acids: incorporation into DNA causes irreversible G2-M cell cycle block in CEM cells (69). An analog of this compound, 6-thio-3-deazaguanine, exhibits even more cytotoxicity than the parent (69).

A new class of nucleoside analogs, the 2-halo-2'- deoxyadenosines, has recently been reported (48). Although little is yet known of their metabolic effects, the compounds are relatively resistant to deamination by adenosine deaminase and so are capable of exerting a prolonged effect compared with that of the parent compound. The drugs cause accumulation of cells in S phase, which is more pronounced with longer exposures and higher concentrations. The loss of cell

viability is associated with a blockade of some process specifically occurring at the initiation of S phase (48). In vivo, the optimum dosage of the 2-chloro compound is lower than that for the 2-bromo compound against L1210 leukemia (48).

New derivatives of N-hydroxy-N-aminoguanidine have been synthesized recently, and have been shown to be active against cancer cells and viral infections through inhibition of ribonucleotide reductase (110). This enzyme catalyzes the 2'-reduction of ribonucleoside 5'-diphosphates; thus, inhibition of the enzyme leads to deoxynucleotide depletion. This in turn causes inhibition of DNA synthesis. A decrease in RNA synthesis is observed, secondary to the inhibition of DNA inhibition (110). The isoquinoline derivative of hydroxyaminoguanidine is the most potent inhibitor of several compounds prepared. The drug causes an accumulation of cells in Go/G1, while M phase cells are not replenished (110). This suggests that the whole of S phase is affected.

Another enzyme, purine nucleoside phosphorylase, is also capable of being inhibited. This enzyme is essential for purine salvage, as it catalyzes the phosphorolysis of guanosine, inosine, and 2'-deoxyribonucleoside derivatives to purine bases. 8-Amino-9-benzylguanine is the most potent inhibitor of this enzyme to date (90). This inhibitor has been used to potentiate the cytotoxicity of 2'-deoxyguanosine for T-cell lymphoproliferative diseases (90), with much less toxicity than occurs with inhibition of adenosine deaminase by 2'- deoxycoformycin (57).

Tiazofurin, 2-α-D-Ribofuranosylthiazole-4-carboxamide (NSC 286193) is an analog of ribavirin and pyrazofurin (32). This drug is phosphorylated by adenosine kinase and/or a cytoplasmic 5'-nucleotidase (32) to form TAD, an analog of NAD (45). An active inhibitor of IMP dehydrogenase (87, 45, 32), the drug causes depletion of cellular guanine pools. An accumulation of IMP results, which, in turn, inhibits HGPRT (87). While the single agent effectiveness of tiazofurin has not been established, the drug shows good therapeutic synergism (45). Another possible use is in sequential chemotherapy: tiazofurin, followed by 3-deazaguanosine or other guanine analogs (87).

Finally, neplanocin A is an antibiotic produced by the actinomycete Ampullariella regularis A11079. An analog of adenosine, the ribose moiety of neplanocin contains a cyclopentane, which may cause the compound to be a poor substrate for ribonucleotide reductase (50). Neplanocin may be activated by adenosine kinase, which would make neplanocin unique, as there are currently no major antitumor drugs available that are activated by this enzyme (50). Neplanocin A selectively inhibits RNA synthesis.

Pyrimidine Analogs

The best known pyrimidine analog is 5-fluorouracil. As with the purines, this drug must be converted to a nucleotide before it is cytotoxic. Fluorodeoxyuridylic acid, the active compound, covalently binds to the active site of thymidylate synthetase in an irreversible inactivation reaction (81). The drug is more toxic to proliferating

than to nonproliferating cells, but is effective against several types of solid tumors. Clinical toxicity is more pronounced in proliferating tissues, such as the gastrointestinal tract and bone marrow (81). Use of (E)-5-(2- bromovinyl)uracil increases the potency of 5-fluorouracil by decreasing its degradation, as the bromovinyl compound inhibits dihydrothymine dehydrogenase (23).

Cytosine arabinoside, ara-C, is a cytidine analog in which the hydroxyl group on C-2 of the furanose is reversed, thus allowing the drug to behave as a 2'-deoxycytidine analog (81). The drug, after activation, inhibits DNA synthesis, but not RNA or protein synthesis, through inhibition of DNA polymerases (81). The drug is very poorly absorbed upon oral administration, and is very rapidly cleared from the bloodstream. Ara-C is a potent myelosuppressive and is used primarily to treat myelogenous leukemia. It is not active against solid tumors (81).

Azacytidine is an analog that has a nitrogen substituted for C-5 of the pyrimidine ring (81). The drug must be incorporated into RNA in order to inhibit protein synthesis. Azacytidine interferes with processing of ribosomal RNA. Reduced production of pyrimidine nucleotides, as a result of inhibition of orotidylate decarboxylase, may contribute to decreased nucleic acid synthesis (81). The drug is more effective against proliferating cells, and is used primarily to treat acute myelogenous leukemia. The deoxy analog of azacytidine is currently in clinical trials (104). After conversion to a nucleotide by deoxycytidine kinase, the drug is incorporated into DNA, where it inhibits DNA methylase. The resulting hypomethylation of DNA has been

associated with activation of gene expression and induction of cell differentiation (104). The mechanism of cytotoxicity of the drug associated with DNA damage may be due to the instability of the drug incorporated into DNA (104).

Ara-AC is an analog of both Ara-C and azacytidine, combining the arabinofuranosyl moiety of ara-C with the triazine base moiety of azacytidine (20). The drug closely resembles ara-C in activity, and exhibits antitumor activity in a wide variety of tumors, including three human tumor xenografts. The drug also shows minimal cytotoxicity to normal murine bone marrow progenitor cells (20).

Cyclopentenyl cytosine has recently been shown to be a potent inhibitor of replication of KB, L1210, and HT-29 human colon carcinoma cells. In addition, the drug induces differentiation of human promyelocytic leukemia cells at concentrations as low as 0.1 μM (71). Marked reductions in intracellular CTP concentrations suggest that inhibition of CTP synthesis is a major mechanism of action. The drug is neither cleaved by uridine phosphorylase nor deaminated by a deaminase, which suggests that a prolonged duration of action is possible (71).

Finally, a new agent, 6-fluoro-2-(2'-fluoro-1,1'-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt, has been shown to inhibit de novo pyrimidine nucleotide biosynthesis (15). The water soluble compound inhibits dihydroorotate dehydrogenase, the fourth enzyme in pyrimidine biosynthesis leading to UMP. The drug causes depletion of pyrimidine nucleotides, with cell death most likely due to inability to synthesize RNA and/or DNA (15). This mechanism of

action is unexpected, as the structure does not resemble known inhibitors.

DNA COMPLEXORS

Compounds that interact with DNA through binding or intercalation are classified as DNA complexors. Many are antitumor agents or antibiotics, and many contain an anthracycline structure. Doxorubicin (adriamycin) is perhaps the best known example of this class.

Adriamycin and daunomycin are believed to act primarily by intercalating base pairs of DNA with subsequent inhibition of DNA-dependent RNA synthesis. These anthracycline drugs are limited by cumulative cardiotoxicity (56). Upon exposure of drug to tumor cells, an initial ionic attraction occurs which is followed by insertion of the hydrophobic region of the drug into the phospholipid region of the cell membrane (11). An increase in membrane fluidity results. Doxorubicin accumulates in the nucleus to a concentration of about one drug molecule per nine base pairs of DNA. NMR studies demonstrate that rings B and C are stacked with the base pairs, while ring D is not; the sugar residue lies in the minor groove of the DNA. Many effects have been noted as being due to either the DNA damage or the changes in the membrane fluidity (11).

Over 500 analogs or derivatives of doxorubicin have been prepared, but only those which have been reported in the recent literature will be discussed here.

Aclacinomycin A is the most active of the aklavinone glycosides against L1210 leukemia. It is orally absorbed, less cardiotoxic,

nonmutagenic, and does not cause alopecia, all of which are improvements over doxorubicin (11). The drug binds to DNA, leading to an accumulation of cells in the G2 and M phases. Interestingly, RNA synthesis is preferentially inhibited by aclacinomycin (11).

Nogalomycin is a polyhydroxy anthraquinone (68) that inhibits nucleolar RNA synthesis more than chromosomal RNA synthesis (11). This compound has not been utilized, however, due to its excessive toxicity in animals. 7-O-Methylnogarol (menogaril), a semisynthetic anthracycline derived from nogalomycin, is the most promising of several analogs (24). Unlike doxorubicin, menogaril is a weak inhibitor of DNA and RNA synthesis and causes no inhibition of RNA polymerase even at highly lethal concentrations (24,25). Menogaril binds weakly to DNA, compared to doxorubicin, yet is still cytotoxic. Menogaril accumulates in the cytoplasm (25). There is a dose-dependent inhibition of progression through S phase with a complete block in the G2 + M phase (1,25). Cell cytotoxicity studies indicate that menogaril is most lethal in early G1, S, and G2, while cells in late G1 are much less sensitive (25).

A trifluoroacetyl substitution on the amino group of the sugar residue converts to the doxorubicin analog AD-32. This compound has been extensively studied. AD-32 is a non- intercalating drug that is less cardiotoxic than doxorubicin. The drug inhibits nucleic acid synthesis, but it has been suggested that this is due to the inhibition of thymidine uptake by the cell (11,77). It is not very soluble; however, AD-143 is a more soluble analog of AD-32. AD-143 selectively inhibits eukaryotic RNA polymerases I and II at lower

concentrations than those necessary to effect similar inhibition of α-DNA polymerase (77).

Cyclic derivatives at the amino group of doxorubicin have equivalent or greater potency as cytotoxic agents. One such compound, 3'-deamino-3'-(3-cyano-4-morpholinyl)doxorubicin, is 600 times more potent than doxorubicin towards P388 tumors in mice (11). This agent does not produce cardiotoxicity at therapeutically efficacious doses, and there is no cross resistance to doxorubicin (109). Structure-activity studies indicate that the morpholinyl moiety, but not the cyano group, is important in the inhibition of total RNA synthesis. Also, the morpholinyl-ring containing compounds are markedly more lipophilic than doxorubicin, resulting in increased cellular transport (96).

Other analogs of interest include epirubicin, esorubicin, and idarubicin. Epirubicin is the 4'-epimer of doxorubicin, and has equal activity but less cardiotoxicity than the parent compound (11,77). Esorubicin, the 4-deoxy analog, is also less cardiotoxic, but has reduced DNA-binding properties (11), and about twice the myelosuppression observed with doxorubicin (84). Idarubicin, 4-demethoxydaunorubicin, is more potent, less cardiotoxic, and shows equivalent DNA binding as the parent compound. In addition, the drug is orally active (11).

One possible technique used to enhance the cytotoxicity of doxorubicin and esorubicin is the use of amiodarone. Amiodarone is an effective anthracycline efflux blocker which acts to prolong the accumulation of doxorubicin inside the cell, thus helping to overcome

resistance (14). The drug is an iodinated benzofurane derivative that is relatively nontoxic. An antiarrhythmic compound, amiodarone has been used in Europe for over 15 years in the treatment of angina pectoris and ventricular arrhythmia (14).

Another compound, hemin, has been shown to preferentially protect hematopoietic cells from adriamycin induced cytotoxicity (101). This could be useful for protecting bone marrow cells during anthracycline treatment of non-hematopoietic malignancies.

Other drugs which complex with DNA cause strand scission. Bleomycin is probably the best example. DNA strand scission is a major mechanism of action of the bleomycins, and this action is enhanced by free-radical-producing systems such as sulfhydryl compounds or other oxidizing or reducing agents (13). Strand scission leads to the formation of nucleoside-derived base N- propenals, of which the thymine and adenine propenals are found to be highly cytotoxic to a variety of tumor cells in culture (72). Bleomycins inhibit cell growth during the G2 phase near the S-G2 boundary (72). Bleomycin is nonmyelosuppressive (56); however, the acute toxicities associated with the drug are dermatitis and allergy, and the dose-limiting chronic toxicity is pulmonary fibrosis (13). Structure-activity test results indicate that the length of the terminal amine is of significance: although not involved in strand scission, the terminal amine may be involved in drug penetration into cells (13).

Other drugs cause strand scission through an effect on the enzymes DNA topoisomerase I or II. Topoisomerase I modifies DNA

tertiary structure and is regarded as critical to DNA replication and gene expression. It has been shown to be inhibited by camptothecin, in vitro. Topoisomerase II, however, is not effected (47). Camptothecin, an alkaloid from Camptotheca accuminata, is a potent cytoxic agent active against a number of experimental murine neoplasms (33). The analog, 10- hydroxycamptothecin, has been reported to have significant clinical antitumor activity in China (9). The biochemical effects reported include DNA strand scission, and inhibition of DNA and RNA biosynthesis (46).

Topoisomerase II reversibly cuts double stranded DNA and becomes covalently linked to the 5' termini at the break site via phosphotyrosyl bonds. Several drugs stabilize this DNA-enzyme complex, resulting in increased DNA scission and concomitant inhibition of the rejoining reaction (97).

One drug which appears to exert its cytotoxic effects by inhibition of topoisomerase II is VP-16 (etoposide) (62). The compound is a semisynthetic epipodophyllotoxin that exhibits cell cycle phase specific cytotoxicity and enhanced effectiveness with increasing duration of drug exposure. The drug delays cell transit through S phase and produces cell cycle arrest in the late S-early G2 phase, but is most effective when cells are in S or G2 phase (93). It has been proven effective against several human malignancies (93). Other work has shown that putrescine inhibits VP-16-induced single strand breaks and associated cytotoxic effects, while difluoromethyl-ornithine (DFMO), which depletes intracellular putrescine and

partially reduces intracellular spermidine (64), acts to produce synergistic cytotoxic effects when combined with VP-16 (26).

Amsacrine, a 9-anilino-acridine compound, is active against leukemia and lymphoma, but its activity in solid tumors is insufficient to warrant further trials. Amsacrine binds to DNA by intercalation and causes single strand and double strand DNA breaks by effecting DNA topoisomerase II (100). CI-921 is a 4- (N-methyl-carboxamide)-5-methyl derivative of amsacrine. The compound is as potent as amsacrine against murine leukemia, but significantly more potent against several murine and human lung, colon, breast, and melanoma cell lines, and against Lewis lung carcinoma, B16 melanoma, colon 38, and MX-1 axenografts in vivo (100). Similar to VP-16, CI-921 slows the transit of cells through S phase, with an eventual accumulation in G2. At subcytostatic concentrations, cell transit is slowed through late S-G2 phases(100).

Nafidimide, a benzisoquinolinedione compound, is currently in Phase I clinical trials. It, too, produces single strand and double strand DNA breaks and DNA-protein crosslinks through effects on topoisomerase II (4).

Two antibiotics from Streptomyces strains, auromomycin and neocarzinostatin, also cause DNA strand scission, but direct involvement with topoisomerases I or II has not yet been shown. Auromomycin is a two component molecule consisting of a fluorescent chromophore and a low molecular weight apoprotein (85). Similarly, neocarzinostatin is made up of an acidic single chain polypeptide and a nonprotein chromophore (76). In both cases, the chromophore is

responsible for the DNA damaging activity, antitumor activity, and cytotoxic activity of the compound (85,76).

MITOTIC INHIBITORS

There are several compounds isolated from natural products which inhibit mitosis in one manner or another and so have been of value in cancer chemotherapy.

The Vinca alkaloids, vinblastine, vincristine, vinleurosine, and vinrosidine, are derived from the white flowered periwinkle Vinca rosea (68). These compounds cause arrest of mitosis in metaphase through interference with the mitotic spindle. Vinblastine and vincristine are also known to restrict the synthesis of transfer RNA (68). The Vinca alkaloids inhibit microtubule assembly and induce tubulin oligomer formation (7). Vinblastine inhibits tubulin-dependent GTP hydrolysis (7). However, neurotoxicity limits the use of vincristine, while leukopenia limits vinblastine (56).

Colchicine, from the autumn crocus, also interferes with spindle proteins (68). Colchicine stimulates tubulin-dependent GTP hydrolysis even as it inhibits the microtubule assembly reaction associated with GTP breakdown (7).

The ansamacrolide maytansine is derived from the East African shrub Maytenus ovatus, and has a macrocyclic lactam ring with a bridged aromatic moiety in its structure (102). Maytansine inhibits mitosis in a manner similar to the Vinca alkaloids; however, it has shown disappointing results in clinical trials (102).

Taxol is a novel diterpenoid originally isolated from the stem bark of the western yew Taxus brevifola (108). The compound is a taxane derivative containing a rare oxetan ring in its structure, and is the first compound of this type to show activity against B16 melanoma, L1210 and P388 leukemias, as well as the human MX-1 mammary tumor, CX-1 colon, and LX-1 lung tumor xenografts (111). Taxol promotes microtubule assembly by decreasing the critical concentration of tubulin necessary for microtubule assembly (111). In fibroblasts and other proliferating cell systems, abnormal bundles of microtubules form throughout the cytoplasm, and binding of taxol to the microtubules occurs. Taxol blocks cells in the G2 and M stages of the cell cycle (111).

A new lactone antibiotic, rhizoxin, is a 16-membered macrolide isolated from the plant pathogenic fungus Rhizopus chinensis, which causes rice seedling blight (102). Rhizoxin inhibits mitosis in the same manner as the Vinca alkaloids, and is effective against vincristine- and adriamycin-resistant tumor cells in vitro and in vivo (102).

Synthetic antimitotic agents have also been prepared. Derivatives of 5,6-Diphenylpyridazin-3-one cause rises in the mitotic index which correlates with the cytotoxicity of the drugs (7). Active derivatives stimulate tubulin-dependent GTP hydrolysis and either inhibit tubulin polymerization or induce tubulin oligomer formation, depending on the reaction conditions. The drugs do not interfere with binding of labelled colchicine, vinblastine, maytansine, or GTP, which suggests that a different binding site is involved. Virtually all of

the active compounds have a nitrile at position 4 of the pyridazinone ring. Most also have substituents of varying structure at position 2, but no clear structure-function pattern is yet apparent (7).

OTHER AGENTS

There are several cancer drugs that can not yet be put in one of the other classes due to lack of knowledge of their mechanisms of action. In addition, there are new agents derived from natural products which have not yet been studied extensively.

The cis-platinum compounds have been used with some success to treat cancer. Cis-diamminedichloroplatinum(II) (CDDP) entered clinical trials in 1972. The drug kills cells in all stages of the cell cycle through a persistent inhibition of DNA synthesis (81). The drug binds to guanine at O-6 and N-7 preferentially, but also to adenine and cytosine. The major dose limiting effect is nephrotoxicity. Other effects include myelosuppression and hearing loss (81). CDDP has also shown synergy with a number of other commonly used agents, including nucleosides and analogs. Use of thymidine with CDDP against human colon carcinoma cells results in a 2-log increase in cell killing (89).

An important second generation drug is dichloro(1,2-diaminocyclohexane)platinum(II); however, it is virtually insoluble in water (43). Water soluble compounds are prepared by substituting an organic anionic leaving group, an iminodiacetate, for the chloride ions. These drugs show good antitumor activity, no in vitro cross

resistance to CDDP, and little or no nephrotoxicity. In addition, they are easily synthesized with a high overall yield (43).

Gold complexes have also shown antitumor activity. Gold(I) phosphine complexes are cytotoxic to P388, B16 melanoma, and mammary adenocarcinoma in vivo (8). The compounds inhibit DNA, RNA, and protein synthesis within 30 minutes exposure to the drug. This activity may be due to improved delivery of the cytotoxic ligand to the target (94).

A group of bis-dioxopiperazines were developed at the Imperial Cancer Research Fund Laboratory. These analogs of EDTA are synthesized with the rationale that they might be activated after entry into the cell (81). ICRF 159 is water insoluble, but its (+) enantiomer ICRF 187 is more soluble. Use of ICRF 187 with doxorubicin has shown synergistic activity (107) which was dose and schedule dependent. It is possible that ICRF 187 chelates intracellular cations which may modulate the effects of doxorubicin (107).

There are several new natural products which have shown anticancer activity. Spergualin is an antibiotic isolated from cultures of Bacillus laterosporus. The 15-deoxy analog had similar activity in vivo against L1210 leukemia, but was effective at lower doses (80). The mechanisms of action have not yet been defined, but it is known that the effects of spergualin depends on the amine oxidase concentration in the medium (60). Metabolic activation of deoxyspergualin is required for activity. Infusions of the compound in dogs causes a toxic cystitis typical of aldehydes (10).

Illudins are low molecular weight products isolated from certain mushrooms (3). They were screened against rodent tumor models and found to have only a narrow index of activity. They have now been shown to be potent in vitro agents against human tumor cells resistant to other known anticancer drugs, and may be preferentially active against leukemias (54). On a molar basis, illudin S is as toxic to CEM, 8392, and HL-60 cells as the plant toxin ricin. Preferential inhibition of tritiated thymidine incorporation and arrest in G1 phase suggests that the compound inhibits DNA sythesis (54).

Agroclavine, an 8-methyl-ergoline type clavine alkaloid, is produced by fungi of the genera Claviceps, Penicillium, and Aspergillus, and by certain species of the dicotyledon family Convolvulaceae (37). It interacts, like the lysergic acid amines, with dopamine and serotonin receptors, and with – adrenergic receptors with lower affinity. Agroclavine has antibiotic activity against some bacteria, and cytostatic activity in the L5178Y murine lymphoma (37). Its in vitro activity is 10-fold higher for lymphomas than for normal splenocytes. The derivative, 1-propyl agroclavine, inhibits thymidine incorporation into DNA, but this is not due to a direct effect on DNA polymerase α or β (29).

L-Canavanine, 2-amino-4-(guanidinooxy)butyric acid, is a nonprotein amino acid synthesized by many leguminous plants. An arginine analog, the compound behaves as an antimetabolite (99). Arginyl-tRNA synthetases are unable to discriminate between canavanine and arginine, thus leading to structural and functional protein aberrations. The compound prolongs the life of mice implanted with

L1210, and produces a significant growth inhibition of solid colon tumors in rats (99).

Metastatic cancer has always been difficult to treat. Recently, it was found that the extent of maturation of oligosaccharide subunits of tumor cell glycoproteins appears to correlate with the malignant potential. This suggests that modification of the oligosaccharide structures on tumor cells may alter the metastatic capacity. Treatment of tumor cells with tunicamycin, swainsonine, or castanospermine causes a substantial impairment of colonization potential such that experimental metastasis is blocked (49). Tunicamycin inhibits the synthesis of an intermediate in the glycosylation process, N- acetylglucosaminylpyrophosphatyl-polyisoprenol (98). Swainsonine inhibits α-mannosidase II in the Golgi (30), and castanospermine inhibits glucosidase I (86). Tunicamycin causes defects in cell adhesion, while the other two do not. This suggests that 1) complete maturation of oligosaccharide chains is necessary, and that 2) carbohydrate modifications affect metastasis in at least two different ways (49).

Certain agents, including a number of low molecular weight planar compounds, can induce differentiation of malignant cells. Tumor cells that have differentiated lose their ability to proliferate, to grow in vitro, and to propagate when transplanted into animals. The rationale behind this technique includes prevention of the onset of malignancies in high risk patients, or the conversion of an existing tumor into a more benign neoplasm with a concomitant loss of growth, invasive, and metastatic properties (95). HMBA, hexamethylene bisacetamide (NSC

95580), a compound related to N-methylformamide and DMSO, is the most potent inducer of differentiation yet defined in this group of compounds (28), but its utility is questionable due to its lack of in vivo activity (16).

Photosensitizers have also received attention of late as possible cancer treatment agents. Hematoporphyrins exhibit selective retention in tumor cells. After i.v. administration of labelled hematoporphyrin derivative, there is a gradual accumulation of radioactivity into tumor tissue (75). Singlet oxygen has been identified as the cytotoxic agent that is probably responsible for the photodynamic destruction of malignant cells exposed to light of the appropriate wavelength and intensity.

Merocyanine dyes have been used as bioelectric membrane probes (5). Merocyanine-540 selectively stains leukemic cells and some immature hematopoietic cells (103). Either the electrical properties or the lipid composition of neoplastic cell membranes allow MC-540 to preferentially penetrate the membrane. Increased uptake of membrane-bound merocyanine-540 occurs upon light exposure, which leads to dye excitation resulting in cell damage and death. How useful these photosensitizers are, in vivo, remains to be determined.

OVERCOMING DRUG RESISTANCE

Drug resistance by tumor cells is a problem which limits the use of many anticancer agents. Ways to overcome resistance have also received attention by researchers. There are several kinds of drugs that overcome resistance by inhibiting drug efflux (63): calcium

channel blockers like verapamil; phenothiazine calmodulin inhibitors like thioridazine, trifluoperazine, and chlorpromazine; liposomotropic amines like chloroquine and propanolol; and some isoprenoids like SDB-ethylenediamine and N- (P-methylbenzyl)decaprenylamine. All of these agents are cationic and amphipathic, and can interact with polar lipids, especially phosphatidylserine (63). Binding to phosphatidylserine can perturb membrane function. Cepharanthine, a bisbenzylisoquinoline alkaloid, is extracted from a menispermaceous plant, Stephania cepharantha Hayata. It too is cationic and amphipathic, and is found to decrease fluidity of various biological membranes (74), thus increasing drug accumulation through inhibition of drug efflux (91).

TARGETING OF DRUGS

Efforts have been made to target antitumor drugs in an attempt to reduce toxicity towards normal cells. One attempt involves the conjugating of a cytotoxic drug to Low-Density- Lipoprotein (LDL) particles (66). An LDL particle contains about 1500 cholesteryl ester molecules surrounded by a polar shell containing apoprotein B. Human cells have receptors for apoprotein B on the surface. After binding, the LDL is internalized and degraded in lysosomes, resulting in a release of cholesterol and amino acids. Freshly isolated human leukemic cells and certain cancer cells in culture have higher LDL receptor activity than the corresponding normal cells (106). The use of LDL carriers might avoid some typical carrier problems such as immunological reactions and rapid plasma clearance (66). One problem

might involve the release of the cytotoxic agent before it can be destroyed in the lysosome.

Liposomes have evolved from simple drug-encapsulated lipid carriers to more sophisticated complexes in which lipid composition (21), size (67,21), and conjugated monoclonal antibodies (17) are all carefully controlled to ensure target specificity. Liposomes composed of particular compounds are fusion-competent when exposed to an acidic environment. Exposure of liposomes to pH 5.0-6.5 (normal pH in endosomes) will cause them to fuse with adjacent membranes (17). In addition, liposomes can modify the ability of a drug to partition into different types of cellular membranes, thus controlling the sites of drug action (21).

Drugs which severely impair membrane function may have potential as antitumor agents if toxicities can be limited. Recently, valinomycin was incorporated into liposomes (21), although it is likely that the drug intercalated into the bilayers (51). The use of cholesterol and anionic lipids is also important for reducing drug-associated toxicities (21).

Synthetic compounds, as varied as they are, demonstrate a variety of anticancer effects. Some rationally designed compounds, such as nucleoside analogs, demonstrate activities which are expected on the basis of their structures. Others exhibit activities different from what was expected, or show no activity at all. The structures of other compounds provide no clue as to their mechanisms of action, as in the case of 6- fluoro-2-(2'-fluoro-1,1'-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt (15).

The synthetic compound, 1-phenyl-3-phenylamino-4-(p-toluenesulfinyl)trans-1,5-hexadiene, is an intermediate in the synthesis of an antibiotic carbapenum (83). Its structure does not resemble other known anticancer agents; however, the structure of the proposed intermediate in solution suggests a mechanism of action similar to the alkylating agents.

In this thesis, the cytotoxicity of the synthetic compound, 1-phenyl-3-phenylamino-4-(p-toluenesulfinyl)trans-1,5-hexadiene (Sulfoxide 1), was examined both in vitro and in vivo. The mechanism of action in vitro was determined by synthesis inhibition studies using radiolabelled precursors. In addition, the toxicity of the compound for normal cells was determined. Additional studies on the cytotoxicity of a p-chloro analog, and liposome delivery system of Sulfoxide 1 were also completed. Results herein suggest that Sulfoxide 1 is more cytotoxic towards proliferating than nonproliferating cells, and that this cytotoxicity occurs through inhibition of DNA and/or RNA synthesis.

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II.

In Vitro Cytotoxic Activity of 1-Phenyl-3-phenyl amino-4-(p-toluenesulfinyl)-*trans*-1,5-hexadiene

ABSTRACT

A novel anticancer drug, 1-phenyl-3-phenylamino-4-(p-toluenesulfinyl)-trans-1,5-hexadiene has been synthesized and found to have in vitro cytotoxicity against P388 (LD_{50}= 30 µg/ml) and L1210 (LD_{50}= 29 µg/ml) murine leukemia cells in culture. The LD_{50} compared favorably with that for doxorubicin. The compound was more cytotoxic to P388 tumor cells than to normal mouse splenocytes. The compound inhibited the uptake of both tritiated thymidine (42% inhibition) and tritiated uridine (24% inhibition) after 3 hours of incubation when used at 5 µg/ml. No effect on uptake of tritiated leucine was observed during this time period. The compound was cytotoxic to normal mouse splenocytes which had been stimulated to divide by the mitogen concanavalin A. No effect was found on normal, non-dividing splenocytes. These results suggest that this novel compound is cytotoxic to leukemic cells or other rapidly dividing cells through inhibition of DNA and/or RNA synthesis.

INTRODUCTION

As part of a continuing study of stereospecific addition reactions of sulfinylallyl anions with ambident electrophiles (1) and their application in the asymmetric total synthesis of antibiotic carbapenums (2) and pyrrolizidine alkaloids, 1-phenyl-3-phenylamino-4-(ptoluenesulfinyl)-trans-1,5-hexadiene was synthesized from cinnamylidene aniline (Fig. 1). This sulfoxide undergoes an elimination reaction in solution to form aniline and a conjugated triene, which may act as a Michael acceptor (3) of a nucleophile from DNA, RNA, or protein. The triene may act as a DNA crosslinker similar to the mitomycins (4).

Preliminary testing of the drug has involved in vitro cytotoxicity assays against the P388 and L1210 murine cell lines; comparison of cytotoxicity with those of prednisone and doxorubicin; DNA, RNA, and protein inhibition assays; time course of DNA and RNA inhibition studies; and cytotoxicity assays against normal cells (splenocytes). Results of this work are reported herein.

MATERIALS AND METHODS

Drugs and Chemicals. [methyl-^{3}H] thymidine (56 Ci/mmol), [5,6-^{3}H] uridine (50 Ci/mmol), and [2,3,4,5-^{3}H]-L-leucine (110 Ci/mmol) were purchased from ICN Radiochemicals, Irvine, CA. Prednisone, doxorubicin, sodium bicarbonate, and Con A (Type IV) were obtained from Sigma, St. Louis, MO. RPMI 1640 powdered media and penicillin-streptomycin solution (10,000 units/ml and 10,000 µg/ml) were purchased from KC Biologicals, Lenexa, KS. FCS was obtained from

Hazleton-Dutchland, Denver, PA. DMSO was from Burdick & Jackson Laboratories. Phosphate buffered saline, (PBS), pH 7.2, contained 137 mM NaCl, 6mM Na_2HPO_4, and 1.5 mM KH_2PO_4 and was sterilized by autoclaving. Cinnamylidene aniline, cinnamylidene p-chloro aniline, Sulfoxide 1, and the p-chloro analog of Sulfoxide 1 were prepared by DHH as described in (1).

Animals. Balb/c mice were originally obtained from The Jackson Laboratory, Bar Harbor, ME, and propagated in-house. Mice were housed in plastic cages under standard laboratory conditions with free access to food and water.

Cell Culture. Cell lines were originally obtained from the American Type Culture Collection, Rockville, MD, and propagated in house. P388 and L1210 cell cultures were maintained in RPMI 1640 media, pH 7.2, supplemented with 10% heat-inactivated FCS, 2 g/l sodium bicarbonate, 100 IU/ml penicillin, 100 µg/ml streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cells were passed regularly into fresh media at 3 day intervals, and only actively growing cultures in the logarithmic stage of growth were used in experiments. Cell number and viability were determined by hemocytometer counting using trypan blue dye exclusion.

Cytotoxicity Assays. P388 or L1210 cells (5×10^6 cells/well) were cultured in RPMI 1640 with concentrations of 20, 24, 32, 40, or 48 µg/ml of Sulfoxide 1 in a final volume of 2 ml/well in 24 well plates for LD_{50} determinations. After 24 and 48 hours, samples were withdrawn for cell number and viability determinations. Cells were also cultured with prednisone dissolved in 6% DMSO and with

doxorubicin dissolved in PBS at varying concentrations for LD_{50} determination.

In another experiment, P388 or L1210 cells (7.5×10^5 cells/ml) were cultured in RPMI 1640 with 10, 20, 25, 30, or 40 µg/ml of Sulfoxide 1 or the p-chloro analog of Sulfoxide 1 for LD_{50} determinations after 24 hours as above.

Incorporation of Labeled Precursors. P388 or L1210 cells (1.5×10^5 cells) were cultured in RPMI 1640 with 1, 2, 4, or 6 µg of Sulfoxide 1 in a final volume of 200 µl in each of two 96 well plates. One plate was used for counting cell number and viability at 3 and 6 hours after assay set up. The other plate was used for pulse labelling of cells for 3 hours with 1 µCi of either [methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine, or [2,3,4,5-^{3}H]- L-leucine, after which the cells were harvested onto Titertek GFA filters (Flow Laboratories, Rockville, MD) using a suction filtration/ water wash Titertek cell harvester. For the protein assay, the cells were precipitated with TCA (10% TCA final concentration) onto Whatman 3 mm chromatography paper and washed with 95% ethanol. The filters were allowed to dry before counting in a Beckman LS 3801 liquid scintillation counter. Results were calculated for comparison as cpm/10^5 viable cells.

Time Course of DNA and RNA Inhibition. L1210 cells (1.5×10^5 cells/well were plated into 96 well plates for a simultaneous DNA/RNA assay. At 30 minute intervals between 0 and 6 hours, 6 µg of Sulfoxide 1 in 6 µl of DMSO, or 6 µl of DMSO alone was added to the appropriate wells. The cells were pulse labelled with either

[methyl-^{3}H]thymidine or [5,6-^{3}H]uridine for the final hour of drug exposure. At time 0, cells were harvested on the cell harvester, dried, and counted on the scintillation counter as previously described. Results were plotted as percent of control cpm vs. time (Figure 2).

Effect on Normal Cells. Cytotoxicity of Sulfoxide 1 for normal cells was determined using splenocytes from the spleens of 6-8 week old BALB/c mice of both sexes. The splenocytes were cultured for 24 hours at 37 ^{o}C in a humidified atmosphere of 5% CO_2/95% air in flasks containing RPMI 1640 supplemented with 10% FCS. Half of the splenocytes were in separate flasks containing Con A at a concentration of 20 µg/ml. After 24 hours, the splenocytes were pelleted, resuspended, and counted to ensure that the same number of viable cells, 3 x 10^5cells/well, were plated. Three different volumes, 1,4, or 8 µl of Sulfoxide 1 dissolved in DMSO at 1 µg/ml, or the corresponding volume of solvent alone were added to the wells with media to a final volume of 200 µl. P388 cells were treated in the same manner as a control. Cell number and viability were determined after 24 hours as discussed above.

In another experiment, spleen cells and P388 cells were treated in the same manner as above, except that cells were pulse labeled for the final three hours with 1 µCi of [methyl-^{3}H]- thymidine before harvesting and counting on the liquid scintillation counter as above.

RESULTS

Properties. 1-phenyl-3-phenylamino-4-(p-toluenesulfinyl)-trans-1,5-hexadiene (Figure 1) is a white powder, soluble in ethanol and DMSO, but insoluble in aqueous solution. Sulfoxide 1, both powder and in solution, is stored at 0 °C. The powder is stable indefinitely. Upon prolonged storage or under acidic conditions, solutions will turn yellow, indicating the presence of the triene rearrangement product.

Cytotoxicity of Sulfoxide 1. Sulfoxide 1 is cytotoxic to both L1210 and P388 cells (Table 1). Diminished viability is observed for both cell lines at 3 hours at the highest concentration of Sulfoxide 1 used. At 6 hours, there is a slight decrease in viability for both cell lines at all concentrations of Sulfoxide 1 used, with the biggest decrease occurring with the L1210 cells (a decrease from 79.37% to 65.90% viability at 42.5 µg/ml Sulfoxide 1). By 24 hours, half or more of all cells cultured with Sulfoxide 1 are dead, and at the highest concentration used, both P388 and L1210 cells were only ≈20% viable. From the LD_{50}, it appears that P388 and L1210 cells are approximately equal in sensitivity to Sulfoxide 1.

Comparison of Sulfoxide 1 Cytotoxicity to that of Prednisone and Doxorubicin. Sulfoxide 1 compares favorably with prednisone and doxorubicin with regard to cytoxicity towards tumor cells in culture (Table 2). Doxorubicin exhibited a nonlinear, biphasic curve (not shown) such that after 48 hours, both 12.5 µg/ml and 60 µg/ml produced 50% cell killing. An LD_{50} could not be determined for doxorubicin at 24 hours. In order to achieve 50% cytotoxicity, a dose of prednisone

more than 27 times that of Sulfoxide 1 was required. The LD_{50} of Sulfoxide 1 for L1210 cells was determined to be 29 µg/ml and the LD_{50} for P388 cells was 30 µg/ml.

Cytotoxicity of the p-chloro analog of Sulfoxide 1. The analog of Sulfoxide 1 showed cytotoxicity comparable to that of Sulfoxide 1. The LD_{50} of the p-chloro analog for L1210 cells was determined to be 68 µM and for P388 cells was 53 µM. The LD_{50} of the parent compound, Sulfoxide 1, for L1210 cells was determined to be 41 µM and for P388 cells 38 µM. These results suggest that P388 cells are more sensitive than L1210 cells to Sulfoxide 1. The addition of a p-chloro group on the phenylamino moiety does not appear to alter the biological activity of the compound. Also, solubility in ethanol is slightly increased, thus making possible a less toxic solvent for drug delivery.

DNA, RNA, and Protein Synthesis. For both P388 and L1210 cells, uptake of [^{3}H]thymidine decreased by 42% at 5 µg/ml during the first 3 hours of the assay (Table 3). Decreases of 65% (L1210) and 85% (P388) were observed at 30 µg/ml Sulfoxide 1 during this same time. The viabilities for both P388 and L1210 cells were greater than 90% at 5 µg/ml, while at 30 µg/ml P388 cells were ≈65% viable and L1210 cells were ≈75% viable. However, the results (cpm's) were normalized for the number of viable cells so that these large decreases in thymidine uptake are due to the higher concentration of Sulfoxide 1 present. Further, more modest decreases in thymidine uptake were observed at 6 and 24 hours (24 hour data not shown). Similar results were obtained using [^{3}H]uridine for both P388 and L1210 cells. An inhibition of 15%

and 24% respectively was observed at 3 hours using 5 µg/ml Sulfoxide 1. There was no inhibition of protein synthesis as shown by uptake of [^{3}H]-L-leucine during the first 3 hours of the assay.

Time Course of DNA and RNA Inhibition. It is possible that Sulfoxide 1 reacts with both DNA and RNA, or, with DNA only, which, in turn, would cause a decrease in DNA-dependent-RNA synthesis. In order to better determine the mode of action of Sulfoxide 1, simultaneous DNA/RNA time course studies were completed. The results in Figure 2 indicate that both DNA and RNA are inhibited most strongly, and to about the same degree, within the first hour of exposure to Sulfoxide 1. A slight variability of inhibition between separate experiments was observed, which may be due to the slightly different percentages of cells in the stages of the growth cycle. DMSO also inhibited the uptake of labelled nucleotide within the first 1 - 1.5 hours. This effect has been previously noted (15). However, even though there is inhibition due to DMSO, there is enhanced inhibition when Sulfoxide 1 is present. After 1.5 hours, DNA and RNA were inhibited by DMSO to 48.7 and 50.2 percent of control, respectively, while inhibition by Sulfoxide 1 was to 29.3 and 11.2 percent of control, respectively.

Effects on Normal Cells. In order to determine whether Sulfoxide 1 was cytotoxic to non-tumor cells, and whether mitotic cells were more vulnerable to the effects of Sulfoxide 1, spleen cells were stimulated with mitogens. The T cell mitogen Con A was used to stimulate half of the splenocytes for 24 hours prior to exposure to Sulfoxide 1 (Table 4). After a further 24 hours, the splenocytes cultured with and without Con A were ≈60% viable in the absence of

Sulfoxide 1 and DMSO. A small decrease in viability was observed with increasing Sulfoxide 1 concentration, such that at 40 µg/ml the splenocytes were 45% viable. There was very little difference in the observed viabilities of splenocytes cultured with and without Con A. It appeared that the mitogen Con A did not have an effect on the cytotoxicity of Sulfoxide 1 towards the spleen cells, as measured by trypan blue dye exclusion. It is unlikely that the spleen cells cultured with Con A were no longer in the process of dividing during the time of Sulfoxide 1 exposure, since the results of a preliminary experiment indicated that 48 hours exposure to 20 µg/ml Con A was optimal (See Chapter 3). The DMSO effect on cell viability was negligible compared with the effects of Sulfoxide 1.

In Table 5, however, a major difference in the uptake of tritiated thymidine by cells treated with and without Con A is apparent. Spleen cells which were not treated with Con A incorporated similar amounts of tritiated thymidine after exposure to Sulfoxide 1. Spleen cells treated with Con A, however, were more susceptible to both DMSO and Sulfoxide 1. At the highest two concentrations used, the spleen cells exposed to Sulfoxide 1 incorporated only 1.6% and 6.4% as much labelled thymidine, respectively, as the cells exposed to DMSO. P388 cells were also susceptible to both Sulfoxide 1 and DMSO, although the cells treated with Sulfoxide 1 incorporated only 34– 42% as much thymidine as those cells treated with DMSO.

These results suggest that mitotic cells are more vulnerable to Sulfoxide 1 than non-mitotic cells, although due to the small number of counts incorporated by the spleen cells not treated with Con A and the variation in the counts of the cells exposed to DMSO, this is not certain.

DISCUSSION

The antibiotic penicillins and cephalosporins are perhaps the best known examples of natural products which contain in their structures the beta-lactam ring (6). In these compounds, the beta-lactam ring is fused with a five- or six-membered sulfur-containing ring. Structural analogs in which the sulfur has been replaced by a carbon atom are known as carbapenums, and are several times more biologically active than the sulfur- containing compound. Sulfoxide 1 is an intermediate compound in the attempted synthesis of a carbapenum (7).

Among the so-called antitumor antibiotics, the bleomycins, for example, contain a mono-beta-lactam ring in their structures (8). The effectiveness of the bleomycins occurs through inhibition of DNA synthesis and/or strand scission (9). Strand scission leads to the formation of base propenals which may be responsible for many of the cytotoxic effects of bleomycin unexplainable by DNA damage alone (9).

The cytotoxicity of Sulfoxide 1 for tumor cells was not unexpected, given that the conjugate addition of nucleophiles and the triene may occur, similar to the reaction of elephantopin with cysteine (3). Sulfoxide 1 may also undergo types of reactions similar

to Mitomycin C, another of the antitumor antibiotics. It has recently been shown that Mitomycin C crosslinks with DNA (10). The reducing conditions influence whether mitomycin C reacts as a mono- or bifunctional DNA alkylating agent. Sulfoxide 1 also has the potential to react as a mono- or bifunctional alkylating agent due to the formation of the triene in solution. Mitomycin C is most active in late G1- early S phase, but is considered to be cycle nonspecific (11). In general, alkylating agents are relatively cell cycle and phase nonspecific, and do not require active cellular replication to be effective in producing cytotoxicity (12). If Sulfoxide 1 acts as an alkylating agent, then perhaps more cytotoxicity towards the normal, non-replicating spleen cells should have been observed.

Doxorubicin, an anthracycline antibiotic, is believed to act primarily by intercalating between base pairs of DNA with inhibition of DNA-dependent RNA synthesis (11). Doxorubicin, however, is less toxic to normal spleen cells than to leukemic spleen cells (13). The possibility remains that Sulfoxide 1 acts in this manner.

The synthetic compound 1-phenyl-3-phenylamino-4-(p-toluenesulfinyl)-trans-1,5-hexadiene (Sulfoxide 1) has been shown to be cytotoxic to tumor cells <u>in vitro</u>. The cytotoxicity is observed within 3 hours of exposure to the drug. After 24 hours exposure to greater than 40 µg/ml Sulfoxide 1, P388 and L1210 leukemia cells decrease to 20% viability, and after 48 hours exposure, viability decreases to less than 10%.

Sulfoxide 1 is not soluble in aqueous solution, necessitating the use of other solvents. DMSO was chosen because of its low

cytotoxicity when used in small quantities, and because Sulfoxide 1 showed marked cytotoxicity for tumor cells when dissolved in DMSO. DMSO is known for its ability to penetrate membranes (14), and may aid in the uptake of Sulfoxide 1 into the cell through the transient permeabilization of the cell membrane which thus allows the uptake of compounds normally excluded by the membrane. This suggests the need for a liposome or other drug delivery method for *in vivo* testing against tumors.

In order to determine the mechanism of cytotoxicity of Sulfoxide 1, its effects on the synthesis of DNA, RNA, and protein were tested. DNA synthesis was inhibited more than RNA synthesis during the first three hour time block, while protein synthesis was not inhibited at all. This suggests that the cytotoxicity of Sulfoxide 1 is due to inhibition of DNA synthesis. The inhibition of RNA synthesis may be a direct consequence of this DNA inhibition, or it may be the result of Sulfoxide 1 reacting directly with the RNA. There is no clear indication of how Sulfoxide 1 affects protein synthesis after the initial three hours.

In order to better determine whether Sulfoxide 1 reacts with DNA, RNA, or both, simultaneous DNA/RNA synthesis inhibition assays were performed. The results indicate that both DNA and RNA are inhibited most strongly, and to about the same degree, after one hour of exposure to Sulfoxide 1. This suggests that Sulfoxide 1 reacts with both DNA and RNA. The differences in the amount of inhibition of DNA and RNA after three hours seen in the previous experiment may be due to the differences in the cell populations of actively growing cells

on different days. This would also explain the slight variability in the degree of inhibition after one hour seen in the simultaneous assay performed on different days. In order to determine if Sulfoxide 1 exhibits phase specificity, or whether Sulfoxide 1 is nonspecific but is more cytotoxic in a particular phase, studies using synchronized cells are suggested.

Because Sulfoxide 1 inhibited both DNA and RNA synthesis, it is probable that protein synthesis is also inhibited, as a consequence of the DNA and RNA inhibition. A different method for harvesting the cells from the proteing synthesis inhibition assay may be successful in detecting this time-delayed inhibition.

The results of testing against spleen cells in Tables 4 and 5 suggest that Sulfoxide 1 may not be as cytotoxic to normal, non-dividing cells as it is to leukemic cells. The results also suggest that 1) Sulfoxide 1 may have a cytostatic effect, depending on the dose, in that the uptake of tritiated thymidine does not correlate with cell death as measured by trypan blue, and/or that 2) trypan blue dye exclusion may not be a valid means of comparing viability between different types of cells due to differences in cell membrane composition which may allow preferential uptake of the dye.

The preference of Sulfoxide 1 for rapidly dividing cells suggests that the compound may be toxic to tissues such as the bone marrow and gastrointestinal tract, which is a common problem with many anticancer drugs.

In summary, Sulfoxide 1 has been shown to be more cytotoxic to murine leukemia cells than spleen cells, and this cytotoxicity occurs

through inhibition of DNA and/or RNA synthesis. Further testing of Sulfoxide 1 will include toxicity in animal models and in vivo testing against tumors.

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Table 1. Cytotoxicity of Sulfoxide 1 for P388 and L1210 leukemia cells.[a]

Sulfoxide 1 (μg/ml)	Percent Viability[b] 3 hr	6 hr	24 hr
P388 cells			
0	93 ± 6.2	93 ± 5.6	95 ± 5.8
12.5	75 ± 12.9	74 ± 5.6	50 ± 8.7
35.0	70 ± 9.7	61 ± 5.3	31 ± 7.6
52.5	60 ± 11.8	53 ± 5.0	21 ± 3.0
L1210 cells			
0	93 ± 7.2	94 ± 5.1	97 ± 2.6
12.5	81 ± 7.2	76 ± 5.3	66 ± 12.5
25.0	78 ± 11.2	76 ± 7.0	41 ± 6.3
42.5	79 ± 8.2	66 ± 7.6	24 ± 5.0

[a] P388 and L1210 cells (7.5×10^5 cells/ml) were cultured with the indicated doses of Sulfoxide 1 for 3,6, and 24 hours at 37 °C. Cell number and viability were determined by hemocytometer counting using trypan blue dye exclusion.

[b] Average ± SEM of 4 (P388) and 5 (L1210) experiments.

[c] Cell counts after 24 hr at the highest concentration used were 1.5×10^5 cells/ml (P388) and 1.8×10^5 cells/ml (L1210).

Solvent effects generally caused less than a 10% decrease in viability after 24 hr at the highest concentration used, and have not been subtracted from these results.

Table 2. LD_{50} Dose Determination.[a]

Drug	P388 cells/ml	LD_{50} (µg/ml)
Prednisone	7.5×10^5	828
Doxorubicin	7.5×10^5	12.5[b]
Sulfoxide 1	5.0×10^5	30 [c]

[a]P388 cells were cultured with varying concentrations of prednisone, doxorubicin, or Sulfoxide 1 for 24 hours at 37 °C. Cell number and viability were determined as in Table 1.

[b]LD_{50} at 48 hours.

[c]LD_{50} for L1210 cells (6.1×10^5 cells/ml) is 29 µg/ml.

All results were calculated from dose response curves using concentrations ranging from 0 to 250 µg/ml (doxorubicin), 0 to 850 µg/ml (prednisone), and 0 to 50 µg/ml (Sulfoxide 1). Results are from 2 or more experiments using 3 well replicates per concentration.

Table 3. Effect of Sulfoxide 1 on DNA, RNA, and Protein Synthesis[a]

Sulfoxide 1 (µg/ml)	cpm/10^5 L1210 cells[b] 3 hr	cpm/10^5 L1210 cells[b] 6 hr	cpm/10^5 P388 cells[b] 3 hr	cpm/10^5 P388 cells[b] 6 hr
DNA Synthesis				
0	68,852 ± 21,234	20,154 ± 3983	78,987 ±17,941	57,697 ± 9509
5	39,171 ± 7,860	8,877 ± 1533	45,334 ± 2,267	19,798 ± 1466
10	45,877 ± 13,609	7,608 ± 491	29,587 ± 3,261	11,861 ± 842
20	25,341 ± 5,818	5,000 ± 1888	12,892 ± 3,249	7,153 ± 1425
30	23,876 ± 3,672	6,356 ± 1868	11,874 ± 580	3,364 ± 801
RNA Synthesis				
0	25,792 ± 3,008	20,861 ± 2380	10,225 ± 1,372	4,839 ± 520
5	19,485 ± 481	15,410 ± 2128	8,682 ± 1,103	3,636 ± 419
10	20,535 ± 2,077	13,575 ± 1908	6,949 ± 336	3,030 ± 287
20	11,488 ± 2,429	8,149 ± 838	6,652 ± 582	3,533 ± 337
30	9,073 ± 649	5,144 ± 411	8,146 ± 1,019	3,385 ± 264
Protein Synthesis				
0	1,146 ± 76	1,605 ± 99		
5	1,063 ± 100	1,639 ± 66		
10	1,237 ± 195	2,061 ± 212		
20	1,225 ± 149	2,788 ± 511		
30	1,312 ± 188	———		

[a]P388 or L1210 cells (7.5 x 10^5/ml) were cultured with indicated doses of Sulfoxide 1 for 3 and 6 hours at 37 °C. Cells were pulsed for 3 hours with 1 µCi of either ^{3}H-thymidine, ^{3}H- uridine, or ^{3}H-L-leucine before harvesting. After counting, cpm's were normalized for the number of viable cells, as determined by cell number and viability counts from a duplicate plate.

[b]Average ± SEM, n = 4

Table 4. Effect of Sulfoxide 1 on Normal Cells[a]

Percent Viability[b]

Volume	Experiment 1		Experiment 2	
(ul)	Sulfoxide 1	DMSO	Sulfoxide 1	DMSO
Splenocytes without Con A				
0	52 ± 10		61 ± 4	
1	57 ± 8	47 ± 9	58 ± 9	50 ± 2
4	57 ± 8	46 ± 7	45 ± 7	65 ± 4
8	44 ± 2	12 ± 2	43 ± 5	71 ± 8
Splenocytes with Con A				
0	66 ± 4		60 ± 11	
1	64 ± 5	63 ±10	56 ± 4	41 ± 5
4	56 ± 6	58 ± 8	47 ± 4	68 ± 4
8	48 ± 4	37 ± 8	41 ± 5	64 ± 4
P388 cells				
0	81 ± 2		97 ± 1	
1	71 ± 5	74 ± 5	92 ± 1	96 ± 1
4	22 ± 4	75 ± 2	23 ± 1	93 ± 2
8	13 ± 3	73 ± 4	23 ± 1	88 ± 4

[a]Spleen cells (1.5×10^6 cells/ml) were cultured with or without Con A (20 µg/ml) and the indicated doses of Sulfoxide 1 (1 µg/µl in DMSO) or DMSO at 37 °C. Cell number and viability were determined as in Table 1.

[b]Average ± SEM, n = 4.

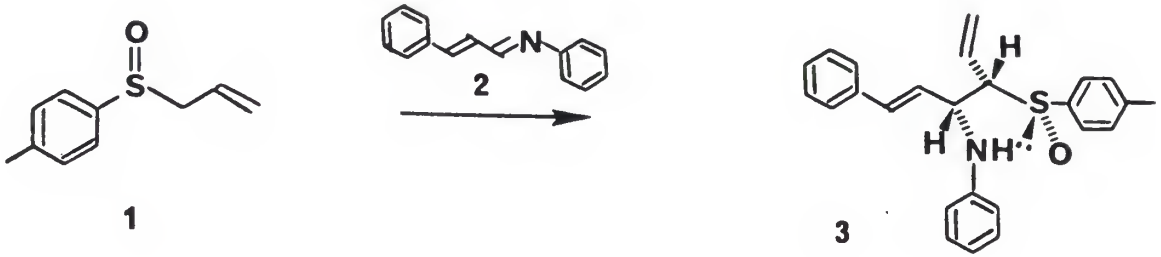
Table 5. Effect of Sulfoxide 1 on Normal Cells[a]

Volume (µl)	cpm's[b] Sulfoxide 1	DMSO
Splenocytes without Con A		
0	545 ± 52	
1	358 ± 32	769 ± 198
4	400 ± 212	181 ± 72
6	378 ± 132	277 ± 177
Splenocytes with Con A		
0	21,213 ± 2648	
1	23,327 ± 3888	18,126 ± 747
4	472 ± 114	7,360 ± 665
6	58 ± 10	3,586 ± 263
P388 cells		
0	25,345 ± 2562	
1	10,430 ± 703	26,890 ± 1746
4	4,301 ± 195	10,124 ± 809
6	2,350 ± 312	6,885 ± 176

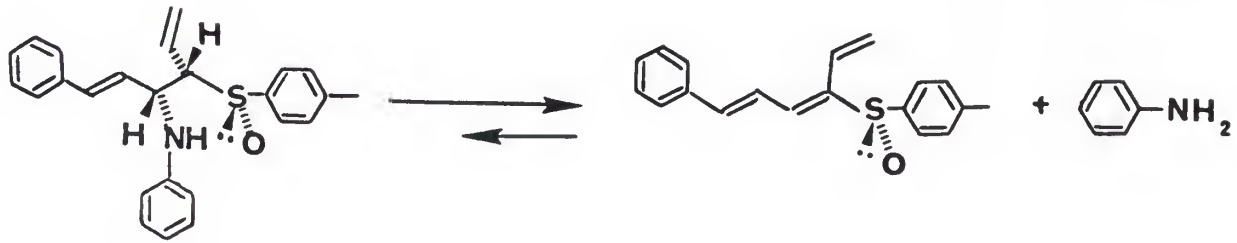
[a]Spleen cells (1.5 x 10^6cells/ml) were cultured with or without Con A (15 µg/ml) and the indicated doses of Sulfoxide 1 (1 µg/µl in DMSO) or DMSO at 37 °C. Cells were pulsed for 3 hr with 1 µCi of [methyl-^{3}H]-thymidine prior to harvest.

[b]Average ± SEM, n = 4.

Fig. 1. Structure of Sulfoxide 1. Sulfoxide 1 (3) was synthesized as a 1,2-α-adduct from (±)p-tolyl allyl sulfoxide (1) and cinnamylidene aniline (2). In solution, sulfoxide 1 undergoes an elimination reaction to produce the triene sulfoxide (4) and aniline.

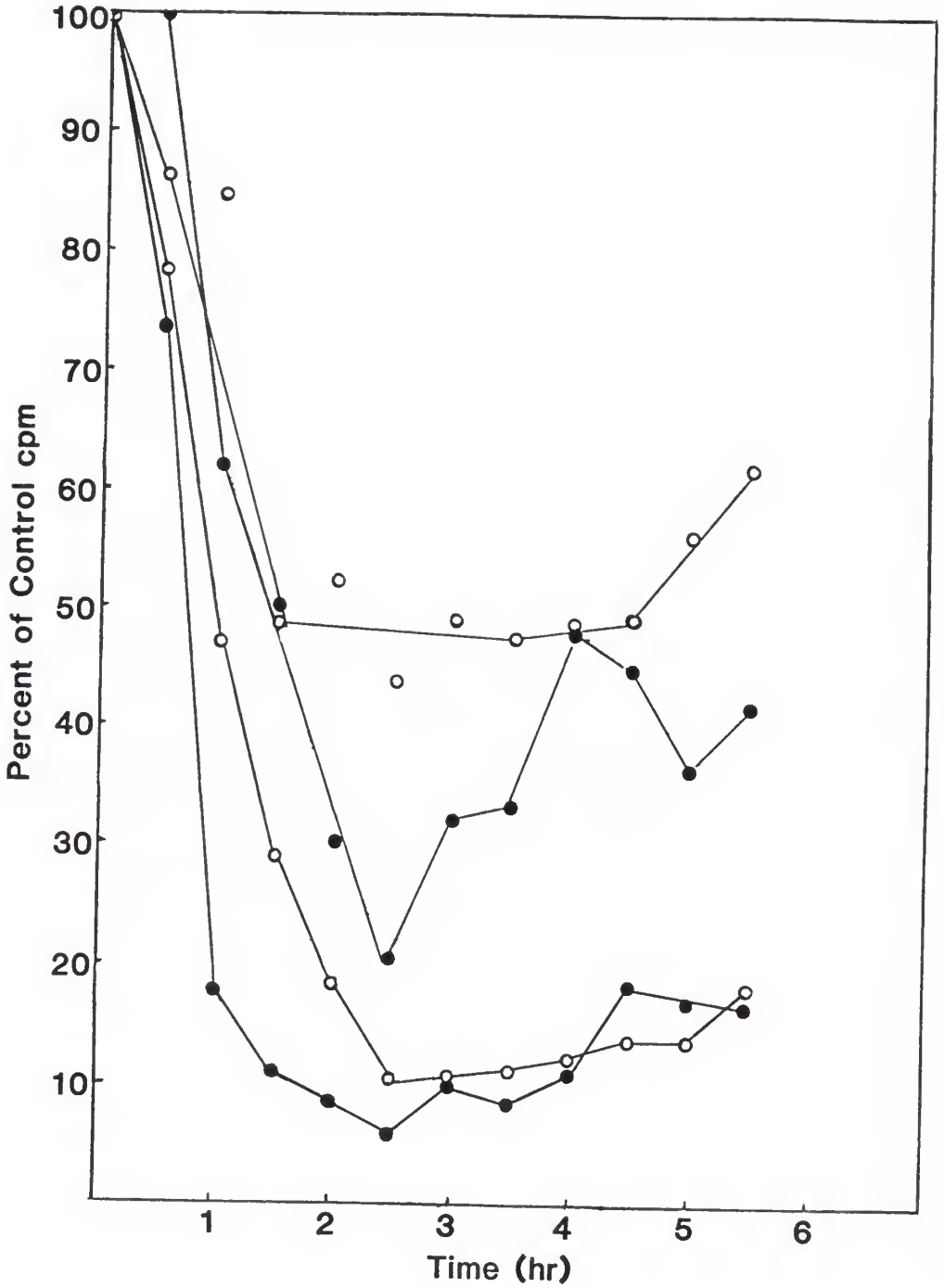


1 3



3 4

Fig. 2. Time Course of DNA and RNA Synthesis Inhibition. L1210 cells (1.5×10^5 cells) were exposed to 6 µg of Sulfoxide 1 in DMSO or 6 µl of DMSO for varying lengths of time, then pulse labelled for 1 hour with 1 µCi of either tritiated thymidine or tritiated uridine. Results are expressed as percentage of control cpm. The upper curves show the inhibition of DNA (-o-) and RNA (-•-) due to DMSO; the lower curves show the inhibition of DNA (-o-) and RNA (-•-) due to Sulfoxide 1.


100
90
80
70
60
50
40
30
20
10
Percent of Control cpm
1
2
3
4
5
6
Time (hr)

III.

Further Studies on Sulfoxide 1

INTRODUCTION

In the previous chapter, the cytotoxicity of Sulfoxide 1 for tumor cells in vitro was shown. Additionally, it was shown that Sulfoxide 1 inhibits DNA and RNA sythesis in a dose-dependent manner, and that this inhibition occurs within the first hour of drug exposure.

To further investigate Sulfoxide 1's DNA binding properties and activity in vivo, additional experiments have been completed. In this chapter, the results of these additional experiments, as well as the results of preliminary work, are reported. These include the solubilization of Sulfoxide 1, the toxicity of Sulfoxide 1 for mice, the effects of Sulfoxide 1 on tumor-bearing mice, the DNA-binding ability of Sulfoxide 1 as measured by equilibrium dialysis, the optimal conditions for Con A stimulation of splenocytes, and a protein assay on tumor cells. The conclusions from each set of experiments are discussed at the end of each section.

Solubilization of Sulfoxide 1

Any new compound must be dissolved in a suitable solvent prior to testing. Water soluble compounds are preferred for ease of testing and, later, for clinical administration. However, most organic compounds are not readily soluble in aqueous solution, necessitating the use of other solvents which, when diluted in aqueous media, will maintain the compound in solution. After the efficacy of the compound has been determined, structural analogs which are designed to increase water solubility may be prepared. Modifications may include the addition of more hydrophilic or polar groups to aid in solubility, or the conjugation of the compound to a water soluble moiety such as a carbohydrate molecule as in the case of doxorubicin (1).

Attempts were made to dissolve Sulfoxide 1 in different solvents as detailed below.

Materials and Methods

Reagents. Sulfoxide 1 was prepared by Dr. D. H. Hua. Fatty Acid Free Bovine Serum Albumin (BSA) was a gift of Dr. R. S. Ochs. DMSO was from Burdick & Jackson Laboratories. L-α- Phosphatidylcholine (PC)(100 mg/ml), and L-α-phosphatidyl-L- serine (PS) (10 mg/ml), were from Sigma. BSA (Fraction V) was from United States Biochemical Corporation, Cleveland, OH. Phosphate buffered saline (PBS), pH 7.2, contained: 137 mM NaCl, 6 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, and was sterilized by autoclaving.

Results and Discussion

Sulfoxide 1 was found to be insoluble in both water and PBS, but was known to be soluble in ethanol (DHH, personal communication). Therefore, 0.6 mg of Sulfoxide 1 was dissolved in 1.0 ml of 95% ethanol. Distilled water was added until a precipitate began to form. The final percent of ethanol was calculated to be 62.5%, and, the Sulfoxide 1 concentration was calculated to be 0.395 µg/µl. This solution was used in a cytotoxicity assay against P388 cells (Figure 1). The results indicated that 62.5% ethanol (v/v), alone, exhibited as much cytotoxicity as Sulfoxide 1, dissolved in 62.5% ethanol. Therefore, this was not the solvent of choice for further cell culture assays.

Sulfoxide 1 was found to be completely soluble in 100% DMSO. The cytotoxicity of DMSO against P388 cells in culture was then determined (Figure 2). Results indicated 94% cell viability using 5% (v/v) DMSO, and H80% viability using 6% DMSO. Subsequent in vitro experiments using Sulfoxide 1 dissolved in DMSO included DMSO-only controls in order to monitor solvent cytotoxicity. Because only very low doses of 100% DMSO can be injected into mice (2), other suitable solvents were sought for in vivo experiments.

Attempts were made to solubilize Sulfoxide 1 by dissolving the compound in 95% ethanol, evaporating the ethanol under N_2 gas, and then adding 1 ml of a solution of 5% BSA dissolved in PBS. This was not successful. Use of 1 ml of Fatty Acid Free BSA gave the same results.

Attempts were also made to dilute solutions of Sulfoxide 1 in DMSO with PBS. A 1% (v/v) solution of DMSO in PBS was possible if the concentration of Sulfoxide 1 was sufficiently low (less than 0.01 µg/µl). However, due to the volume constraints posed by animal injections, more concentrated solutions of Sulfoxide 1 were required. Final concentrations of 20% and 50% DMSO in PBS were insufficient to keep the Sulfoxide 1 in solution.

Liposome Delivery System

Attempts were also made with some success to encapsulate Sulfoxide 1 in liposomes. Liposomes were prepared by dissolving 0.1 to 0.3 mg of Sulfoxide 1 and 50 µl each of PC and PS in 1 ml of $CHCl_3$ (in which Sulfoxide 1 was very soluble), then evaporating under N_2 gas. The dry residue was rehydrated in 1 ml of PBS for 10 min at 25 °C. The test tube was then heated to 60 °C in a water bath and immediately vortexed for 1 min. After centrifugation at 14,000 x g for 10 min in a Sorvall RC2B centrifuge, the liposomes were resuspended in 1 ml of PBS and used immediately. The resuspended liposomes appeared as a white solution, but as no precipitate formed upon standing, the method was presumed successful.

It is apparent that solubility is a major consideration during all phases of compound screening. When compounds are not water soluble, organic solvents of an innocuous nature must be used. The results of testing indicate that Sulfoxide 1 was ineffective in ethanol solution, but much more cytotoxic when dissolved in DMSO. DMSO is known for its ability to penetrate membranes (2); it appears

that Sulfoxide 1 was able to enter the cell when dissolved in DMSO, but not when dissolved in ethanol.

Animal Tumor Model Systems

After the efficacy of the compound was determined in vitro the next step was to determine the effectiveness of the compound in vivo against a panel of different tumors. Further testing was done to determine the toxicity of the compound in animal models.

Sulfoxide 1 was tested in mice in order to determine the LD_{50}, the toxicity to internal organs, and effectiveness against an implanted L1210 lymphoma.

Materials and Methods

Animals. Rd/1e, C3H/HeN, and DBA/2 mice were originally obtained from Jackson Laboratories, and subsequently propagated in-house. Six to eight week old mice of both sexes were used in experiments unless otherwise stated.

Reagents. Sulfoxide 1 was prepared as previously described by Dr. D. H. Hua. Suspensions of Sulfoxide 1 and liposomes were prepared as under the section on "Solubilization".

Cell cultures. L1210 cells were originally obtained from the American Type Culture Collection (ATCC) and subsequently propagated in-house by culturing in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% Fetal Calf Serum (FCS).

Results and Discussion

In the first experiment, six C3H/HeN mice were injected intraperitoneally (i.p.) with 0.1, 0.5, or 1 ml of either 1 mg/ml BSA

in PBS or 6% DMSO (v/v) in PBS. The same mice were reinjected one week later with the same volume of either 5 mg/ml BSA or 10% DMSO. Mice were reinjected 3 days later with 5% BSA or 50% DMSO. Only one death occurred: that of the mouse injected with 1 ml of 50% DMSO. From this it was concluded that 5% BSA and up to 0.5 ml of 50% DMSO would be suitable carriers for injecting Sulfoxide 1 into mice.

In the second experiment, the remaining mice from trial 1 were injected i.p. with Sulfoxide 1 via one of the following: 1 ml of 1% DMSO (8 µg Sulfoxide 1); 0.75 ml of 5% BSA (H30 µg Sulfoxide 1); 0.5 ml of 20% DMSO (80 µg Sulfoxide 1); or 1 ml of 20% DMSO (160 µg Sulfoxide 1). No deaths occurred, and it was concluded that the LD_{50} range of Sulfoxide 1 had not yet been reached. Thus, Sulfoxide 1 does not appear to be highly toxic when administered in vivo.

In the third experiment, 4 week old rd/le mice were injected, i.p., with volumes ranging from 0.1 to 0.35 ml of 50% DMSO containing 125 to 525 µg of Sulfoxide 1. However, these were suspensions; the volume of DMSO, constrained by the results of trial 1, was not enough to hold the higher amounts of Sulfoxide 1 in solution. One death occurred: that of the mouse injected with 525 µg of Sulfoxide 1 in 0.35 ml of 50% DMSO. The mouse injected with the next highest amount of Sulfoxide 1, 375 µg in 0.25 ml, survived. Thus, it was concluded that the range of concentration for LD_{50} determination had been found and was between 375 and 525 µg Sulfoxide 1.

For the next experiment, C3H/HeN mice of both sexes were weighed and divided into six groups of 4 mice each and one group of 7 mice. The groups of 4 mice were injected with 400, 450, 475, 500, 525, and

575 µg of Sulfoxide 1 in a suspension of 50% DMSO. The group of 7 mice was injected with a corresponding volume of 50% DMSO. Twentyfour hours later, only 2 deaths had occurred: one mouse (weight 15.0 g) which had been injected with 450 µg Sulfoxide 1 (30 mg/kg); and one mouse (weight 20.6 g) which had been injected with 0.5 ml of 50% DMSO. These deaths may have occurred from an internal organ being pierced by the needle at the time of i.p. injection. Five days after injection, one animal from each group was sacrificed and examined for gross pathological defects (see below). The remaining animals in the groups previously injected with 400–475 µg were divided into 2 groups and injected with 600 or 650 µg Sulfoxide 1 as above. No deaths occurred after these injections. The injection of 650 µg of Sulfoxide 1 into a mouse weighing 15.2 g corresponds to an injection of 42.7 mg/kg. Although an LD_{50} was not determined, an injection of 42.7 mg/kg was deemed sufficient for declaring Sulfoxide 1 relatively nontoxic to mice.

Thirty days after injection, all mice were sacrificed and examined for gross pathological defects. It was found that 19 of 24 mice had liver necrosis; 7 of 24 had intestinal necrosis; 4 had necrosis at the site of injection; and 1 had a granulomatous sac typical of Type IV hypersensitivity (3). Of the eight mice which had been injected twice, 4 had necrosis on the diaphragm in addition to the liver necrosis, and had slightly enlarged livers. In contrast, those mice which had been injected with 50% DMSO had no observable necrosis or other changes. These results indicate that the liver is

affected by Sulfoxide 1, probably due to the metabolic clearance of the compound by the liver.

In order to determine the efficacy of Sulfoxide 1 against implanted tumors, male and female DBA/2 mice were weighed and divided into three groups of 4 and one group of 2, before i.p. injection with 1×10^5 L1210 cells. One group received i.p. injections of 0.1 mg of Sulfoxide 1 in 50% DMSO on days 1 and 7, one group on days 3 and 10, and one group on days 6 and 13 after tumor implantation. The control group received only tumor cells. Mice were analyzed for weight gain, tumor growth, and survival time. There was no increase in life span (ILS) for any of the mice that received Sulfoxide 1.

In the next experiment, two groups of 2 DBA/2 mice were injected, i.p., with 1×10^5 L1210 cells. Sulfoxide 1 was injected directly into the tumor on the first day of nodule detection, and on days 3 and 8 following the first injection. Again there was no increase in life span. It was concluded that Sulfoxide 1 could not overcome the tumor burden if injections were delayed until the tumor was detectable by palpation.

In the third experiment, two groups of mice were i.p. injected with L1210 cells as before. The test group received i.p. injections of 0.1 mg of Sulfoxide 1 in 0.1 ml of 50% DMSO on days 3 and 6 after L1210 implantation. There was no increase in life span; on the contrary, 2 of 4 test group mice died 2 days before 1 of 3 control group mice died.

At this time it was clear that Sulfoxide 1 was not cytotoxic against L1210 cells in vivo in our system. It was thought that the

delivery system, a suspension of Sulfoxide 1 in 50% DMSO, was inadequate. An in vitro experiment confirmed this suspicion. Wells containing 1.5 x 10^5 L1210 cells and 0, 1, 2, 4, or 6 µg of Sulfoxide 1 as a 1 µg/µl suspension in 50% DMSO were incubated for 6 and 24 hours before determining cell number and viability using trypan blue dye exclusion. All viabilities were above 90%. In contrast, cells which had been exposed to 6 µg of Sulfoxide 1 in 100% DMSO typically showed H70% viability after 6 hours, and 38% viability after 24 hours. It was concluded that suspensions of Sulfoxide 1 in 50% DMSO are not cytotoxic either in vitro or in vivo, and that another drug delivery method was needed in order to continue in vivo testing. It is probable that the Sulfoxide 1 was unable to enter the cell as a solid in suspension.

In the next experiment, two groups of DBA/2 mice were i.p. injected with L1210 cells as before. One week later, the test group of mice received a single 0.2 ml ,i.p., injection of liposomes (see section on "Solubilization" for method of preparation) that were made with 0.1 mg of Sulfoxide 1. There was only a 2 day increase in the Mean Survival Time (MST) of the test group, which was not significant.

For the next experiment, the test group was i.p. injected with 0.1 ml of liposomes once weekly for three weeks, beginning 4 days after tumor implantation. The MST of the test group increased 4 days over that of the control group, but again, was not significant.

In the next experiment, liposomes were, again, i.p. injected into the test group of mice. Beginning one week after tumor implantation, injections were given daily for 5 days, followed by 2 days of no

injections, then 5 more daily injections. The injections of 0.1 ml were directly into the tumor, as before. There was no increase in life span following this protocol. In fact, the tumors of test group mice appeared to grow larger and faster than those of the control group mice. This may have been due to the slow absorption of the fluid in the daily injections.

In the final experiment, two groups of three DBA/2 mice were injected with L1210 cells as before. The test group received once weekly injections of 100 µg of Sulfoxide 1 in 0.1 ml of 100% DMSO, beginning the day of tumor implantation. Once again, there was no increase in life span.

Equilibrium Dialysis

Sulfoxide 1 has been shown to inhibit the uptake of labelled thymidine and uridine. It is suspected that Sulfoxide 1 covalently bonds to nucleophiles from DNA and RNA. In order to determine whether Sulfoxide 1 binds to DNA, a DNA-binding experiment using the technique of equilibrium dialysis (4) was attempted.

Materials and Methods

Reagents. Sulfoxide 1 and PBS were prepared as previously described. DMSO was from Burdick & Jackson Laboratories, and 1,4 dioxane was from Fisher Scientific. Calf thymus DNA (Sigma), 0.5 mg/ml, was dissolved overnight in a 10% DMSO in PBS solution.

Materials. Dialysis tubing was from Spectrapor, Molecular Weight Cut-Off (MWCO) 6-8,000, and VWR Scientific, Inc., 24 Å. Dialysis cells of 1 ml capacity were from Technilab Instruments.

Results and Discussion

In order to follow the progress of an equilibrium dialysis experiment, it is necessary to measure the concentrations of the "bound" vs. "free" ligand very accurately. In order to determine whether this was possible using Sulfoxide 1 as the ligand, the UV spectra between 250 - 350 nm of Sulfoxide 1, 0.1 mg/ml in 10% DMSO, and DNA in 10% DMSO were taken in a Shimadzu UV-120-02 Spectrophotometer (Figure 3). The results indicate that the λ_{max}= 270 nm for Sulfoxide 1, and the λ_{max}= 260 nm for DNA. However, DNA does

not absorb above 320 nm, while Sulfoxide 1 had a measurable absorbance through 350 nm. Thus it was concluded that it would be possible to measure changes in Sulfoxide 1 concentration spectrophotometrically below 320 nm.

A sample of Sulfoxide 1 in 10% DMSO was placed in one side of the dialysis chamber, and the solvent, 10% DMSO in PBS, was placed in the other. The membrane used was from a previously prepared sample, MW cut-off 8,000, which had been preserved in sodium azide and rinsed well in deionized water prior to use. After 24 hr, the absorbance on either side of the membrane was measured between 300 - 330 nm. From the results, it was concluded that 1) Sulfoxide 1 would pass through the membrane; 2) the DMSO would not destroy the membrane; and 3) that more than 24 hr. would be required to reach equilibrium.

A standard curve of Sulfoxide 1 was attempted. During the course of diluting concentrated solutions for absorbance measurements, suspensions resulted, which are very difficult to use as a measure of concentration. At the time, it was thought that during the actual experiment, the DNA would bind enough of the Sulfoxide 1 for the remainder to be held in solution so that concentrations could be measured. Spectrapor membranes (MWCO 6 - 8,000) were boiled in double distilled water, cooled, and split to form a single layer. Pieces were cut to fit the dialysis chambers. As soon as possible after assembling the chamber with a membrane, both sides were filled with the appropriate solution using 1 cc tuberculin syringes. Duplicate cells were assembled with Sulfoxide 1 vs. DMSO/PBS in one cell (control), and the same concentration of Sulfoxide 1 vs. DNA in

DMSO/PBS in the other. Cells were agitated on an orbit shaker at room temperature. After 96 hr., the absorbance on either side of the membrane was measured in the control cell. Results indicated that almost none of the compound passed through the membrane in the control cell, but that some did pass through the membranes of the chambers containing DNA. The sides of the chambers containing DNA were not measured for absorbance, therefore it is unknown whether equilibrium was reached in those cells. It is clear that equilibrium was not attained in the control cells.

In an effort to speed up the equilibrium process, a membrane with larger pore size was used in time trials. Dialysis tubing of pore size 24 A was prepared in the following manner: After heating at 80 - 90 ^{o}C for 1 - 2 hr in 10 mM sodium bicarbonate and 1 mM EDTA, the tubing was then heated at 80 - 90 ^{o}C for 1 -2 hr in double distilled water. The tubing was allowed to soak at room temperature in fresh double distilled water for several hours before being stored in a solution of 50% ethanol in deionized water. The tubing was rinsed in deionized water immediately before use, then, soaked for 4 -24 hr in the buffer in which it would be used; in this case, 10% DMSO in PBS. Chambers were assembled and filled as before, but with 1 ml only to ensure consistent volumes. In order to determine whether DNA could pass through the larger pore size, one cell contained DNA vs. solvent, and the other cells contained 100 µg Sulfoxide 1 vs. solvent for absorbance measurements at 24, 48, and 72 hr. The absorbance measurements indicated that the DNA or the membrane was probably contaminated with some small absorbing ion as there was a very slight

absorbance of the solvent compared with the solvent containing DNA. The Sulfoxide 1 containing cells never reached equilibrium; in fact, the Sulfoxide 1 aggregated as a precipitate in the bottoms of the chambers. The trial was repeated using half as much Sulfoxide 1, with similar results. The trial was repeated a third time using 20% DMSO in PBS as the solvent with similar results.

Solubility trials were made using 10% and 20% 1,4-dioxane in PBS; however, the DNA was precipitated by this solvent.

It was concluded that a DNA-binding experiment using the technique of equilibrium dialysis could not be performed due to the constraints posed by the differing solubility needs: DNA requires a high salt solution in order to minimize the charge effects on either side of a membrane; Sulfoxide 1 requires an organic solvent such as DMSO in order to remain in solution; and, equilibrium dialysis requires solutions, not suspensions, in order to reach equilibrium.

Optimal Conditions for Concanavalin A Stimulation of Normal Mouse Splenocytes

Sulfoxide 1 has been shown to be effective against tumor cells in vitro. In order to determine whether Sulfoxide 1 is more effective against dividing vs. non-dividing cells, a population of cells was chosen for testing which is capable of both states. Spleen cells are made up of both T and B lymphocytes, which exist in a quiescent state until stimulated by antigens. Lymphocytes may also be stimulated to divide through the use of mitogens. The most commonly used mitogens are plant lectins which bind specifically to certain sugar residues on the glycoproteins found on cell surfaces(5). Mitogens are specific: phytohemagglutinin (PHA), from the red kidney bean, and Concanavalin A (Con A) from jack beans, stimulate only T cells, while pokeweed mitogen (PWM) and Bacterial lipopolysaccharide (LPS) are specific for B cells. Mitogenic effects are distributed over a Gaussian curve. Therefore, optimal conditions with respect to mitogen concentration and time of exposure for maximal stimulation must be determined prior to use.

Materials and Methods

Reagents. Con A, Type III or IV, dissolved in RPMI 1640 media and filter sterilized through a 0.22 μm Acrodisc filter (Gelman, Ann Arbor, MI) was prepared fresh for each experiment, and kept on ice until use. [Methyl-^{3}H]-thymidine (50 Ci/mmol) was from ICN

Radiochemicals. Lysing solution contained 280 mM NH_4Cl in 30 mM TrisCl, pH 7.4. PBS was prepared as previously described.

Animals. DBA/2 mice were originally from Jackson Laboratories, and subsequently propagated in house.

Results and Discussion

Spleens were removed from 1 or 2 mice and the splenocytes dispersed in cold PBS. Pelleted cells were lysed with 1 ml of lysing solution for 3–4 min. at room temperature in order to remove red blood cells. Cells were then washed 3X with media containing FCS.

In the first set of experiments, 5×10^5 spleen cells/ well were plated into 96 well plates with 0, 5, 10, or 20 µg/ml Con A Type III in 200 µl final volume. After 24 hours, spleen cells were counted for cell number and viability using trypan blue dye exclusion. The second experiment was set up in the same manner, except that cells were counted after both 24 and 48 hours. It was concluded that this method was not appropriate for determining maximal stimulation by Con A. Consequently, in subsequent experiments cells were pulsed with 1 µCi of tritiated thymidine, as thymidine incorporation provides a better correlation of maximal stimulation to cellular mitotic activity.

In the first experiment, spleen cells were plated as above and cultured with 0, 5, 10, 15, 20, or 30 µg/ml Con A for 24 hr, and pulsed with labelled thymidine for the final 3 hours. Results indicated only a slight gain in mitotic activity over control cells.

In the second experiment, splenocytes were exposed to 0, 5, 10, or 20 µg/ml Con A for 24, 48, or 72 hours. Results (Table 1)

indicated that 48 hours exposure to 20 µg/ml Con A afforded maximal stimulation to spleen cells.

It was necessary to redetermine the optimal time and concentration of Con A necessary for maximal lymphocyte stimulation. By mistake, the wrong bottle of Con A was used, such that, in this experiment, the more highly purified Con A Type IV was used rather than the Type III used previously. Splenocytes were cultured with 0, 5, 10, 15, 20, or 30 µg/ml Con A and pulsed with labelled thymidine as before. Results indicated that 48 hours exposure to 15 µg/ml was optimal. The more highly purified Con A gave much greater stimulation than the Type III Con A used previously. This was beneficial, however, because a greater contrast in results was seen after the cells had been exposed to varying amounts of Sulfoxide 1 (see Table 5, Chapter II).

Protein Assay

In order to determine the amount of protein present in a given number of tumor cells, a protein assay using the Bradford method (6) was performed using bovine serum albumin as a control.

Materials and Methods

Reagents. Bio-Rad Protein Assay Dye Reagent Concentrate was from Bio Rad Laboratories, Richmond, CA. BSA was dissolved in distilled water as a 1 mg/ml solution and used to generate a standard curve.

Cell Culture. P388 cells were originally obtained from the ATCC, and subsequently propagated in house. Because cells are normally cultured in RPMI 1640 media supplemented with 10% FCS, it was necessary to wash the cells several times with PBS to remove all traces of serum proteins.

Results and Discussion

Replicate assays were run on samples containing 1×10^5 cells, 2×10^5 cells, and 1×10^6 cells. Briefly, 0.8 ml of sample was mixed with 0.2 ml of the dye reagent, and the absorbance of the resulting solution was measured on a Shimadzu UV-120-02 spectrophotometer at 595 nm.

From the standard curve generated, 1×10^5 cells contain 3.75 - 4.5 µg protein, 2×10^5 cells contain 6 - 8.75 µg protein, and 1×10^6 cells contain 30.5 - 33.5 µg protein. Because it is difficult to control the exact number of cells in a sample, these ranges reflect

not only the differing amounts of protein in a given number of cells but also the differing numbers of cells in the sample.

SUMMARY

This chapter has described experiments designed to determine the activity of Sulfoxide 1 both as a toxic substance and as an antitumor agent in vivo, and as a DNA-binding compound. In addition, the results of preliminary experiments necessary for further work including the solubilization of Sulfoxide 1, optimal conditions for Con A stimulation of splenocytes, and a protein assay on tumor cells, have been described.

In summary, the results in this chapter have shown:

a) Sulfoxide 1 is soluble in the organic solvents chloroform and DMSO, slightly soluble in ethanol, and insoluble in aqueous solution. BSA, as a carrier system, was unsuccessful. Liposome encapsulation appeared to be successful as a solubilizing system.

b) Suspensions of Sulfoxide 1 are not cytotoxic either in vitro or in vivo.

c) Sulfoxide 1 is not toxic in mice when injected i.p. up to 42.5 mg/kg.

d) Liver necrosis appears to be the major observable toxicity of Sulfoxide 1.

e) In vivo, Sulfoxide 1 was ineffective against our system of implanted L1210 leukemia.

f) Sulfoxide 1 has an absorbance spectrum between 250 -350 nm, past the point of absorbance by DNA.

g) DNA binding by equilibrium dialysis cannot be measured until a better solvent system is defined.

h) The optimal conditions for Con A stimulation of splenocytes is 48 hours at 20 µg/ml (Type III), and 48 hours at 15 µg/ml (Type IV).

i) P388 tumor cells contain the following amounts of protein: 1 x 10^5cells, 3.75 - 4.5 µg protein; 2 x 10^5 cells, 6 - 8.75 µg protein; and 1 x 10^6 cells, 30.5–33.5 µg protein.

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Table 1. Optimal Conditions for Con A Stimulation of Normal Mouse Splenocytes[a]

Con A	cpm[b]		
(ug/ml)	24 hr	48 hr	72 hr
0	3244 ± 467	3120 ± 1097	816 ± 54
5	4965 ± 452	15464 ± 292	6473 ± 1580
10	7548 ± 1089	24090 ± 1227	9889 ± 351
20	6193 ± 96	33191 ± 4628	15471 ± 185

[a]Splenocytes (5×10^5 cells/well) were cultured with the indicated doses of Con A for 24, 48, and 72 hours at 37 °C. Cells were pulsed for 3 hours with 1 uCi of ^{3}H-thymidine before harvesting.

[b]Average ± SEM, n = 4

Fig. 1. Sulfoxide 1 Cytotoxicity Assay. P388 cells were exposed to Sulfoxide 1 (0.395 µg/µl) dissolved in 62.5% ethanol (-●-) and to 62.5% ethanol alone (-o-) for 24 hours.

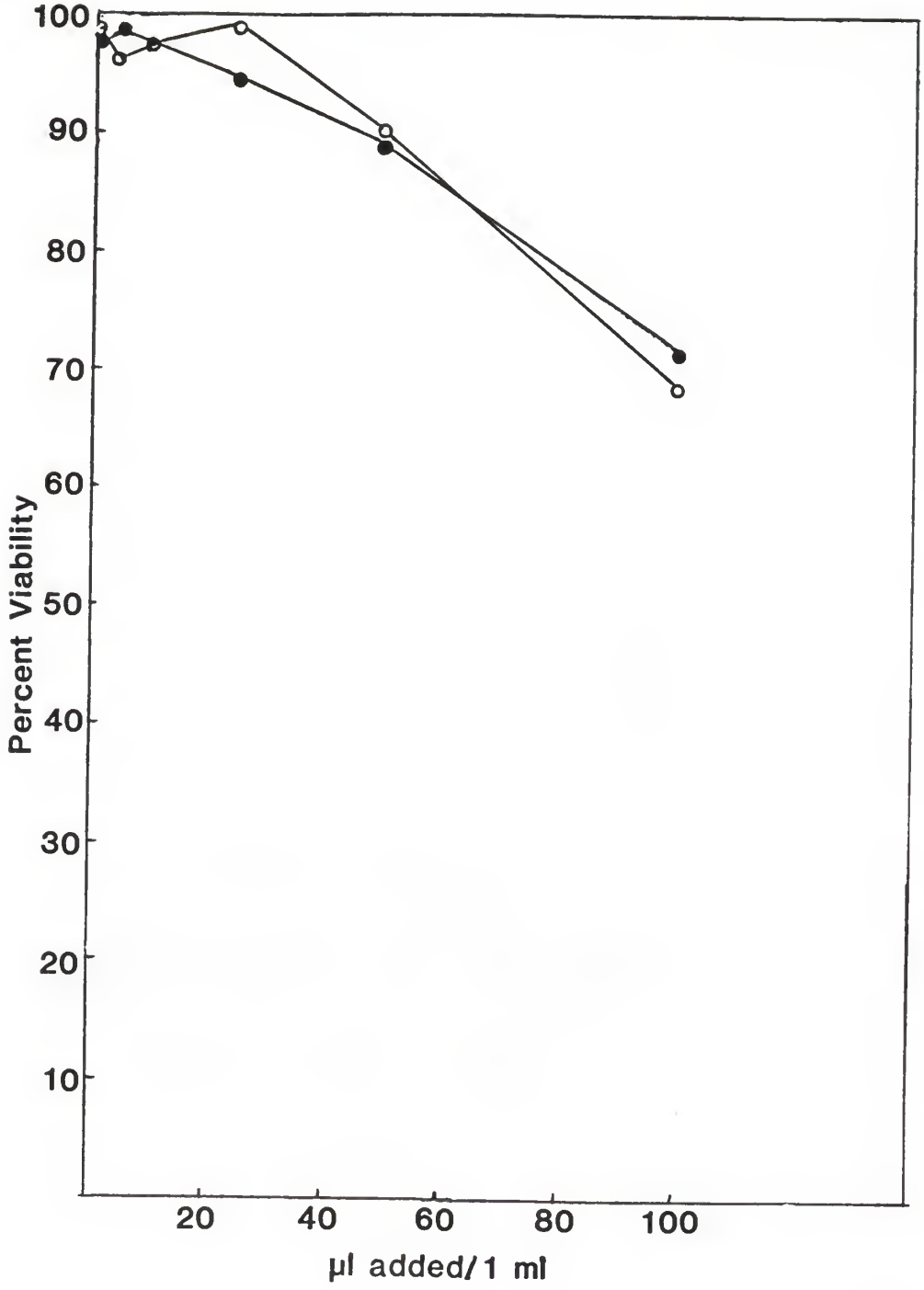

100
90
80
70
60
50
40
30
20
10
Percent Viability
20
40
60
80
100
µl added/1 ml

Fig. 2. DMSO Cytotoxicity Assay. P388 cells were exposed to 1,5, 10, and 20% DMSO for 24 hours.

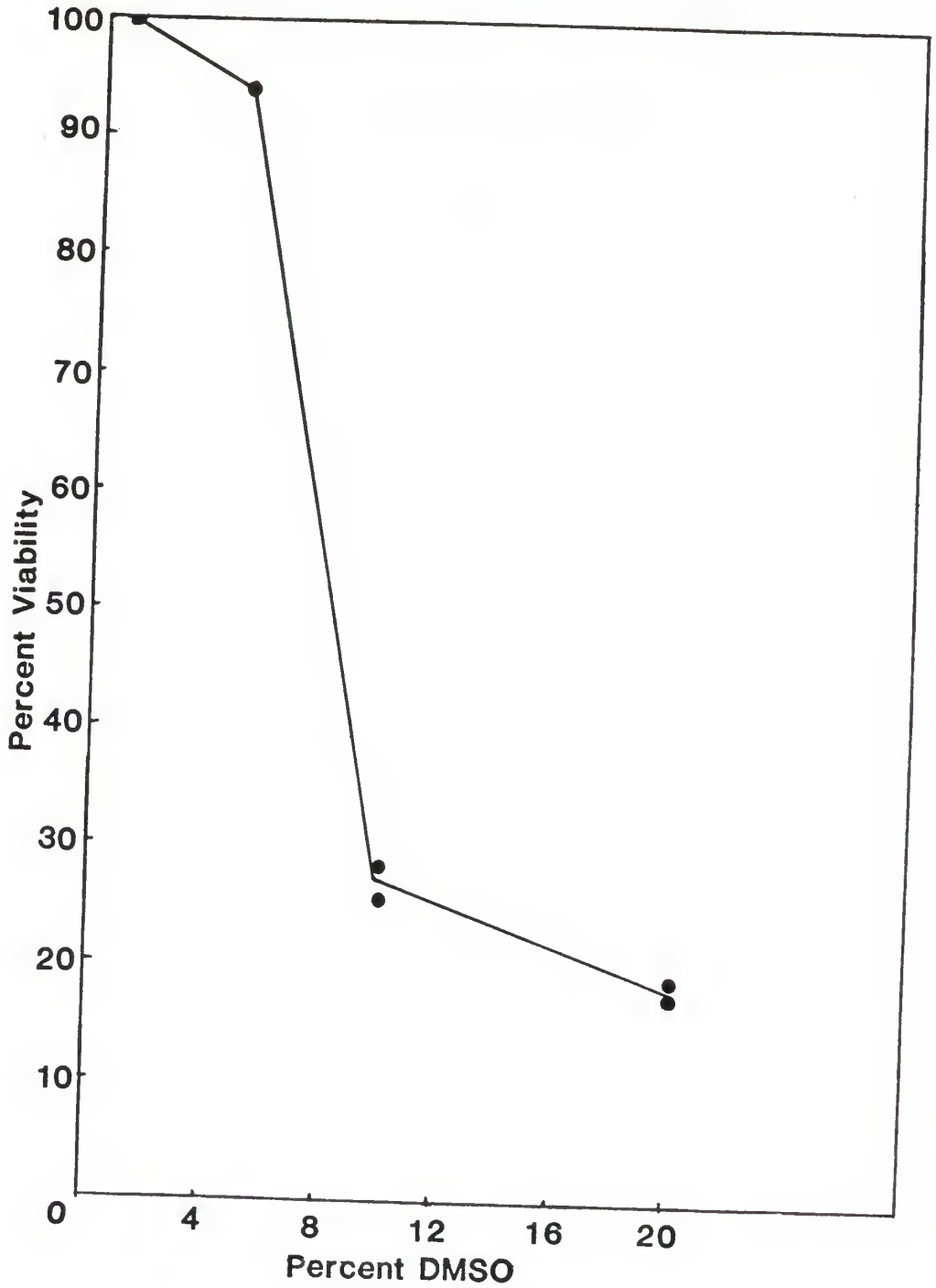
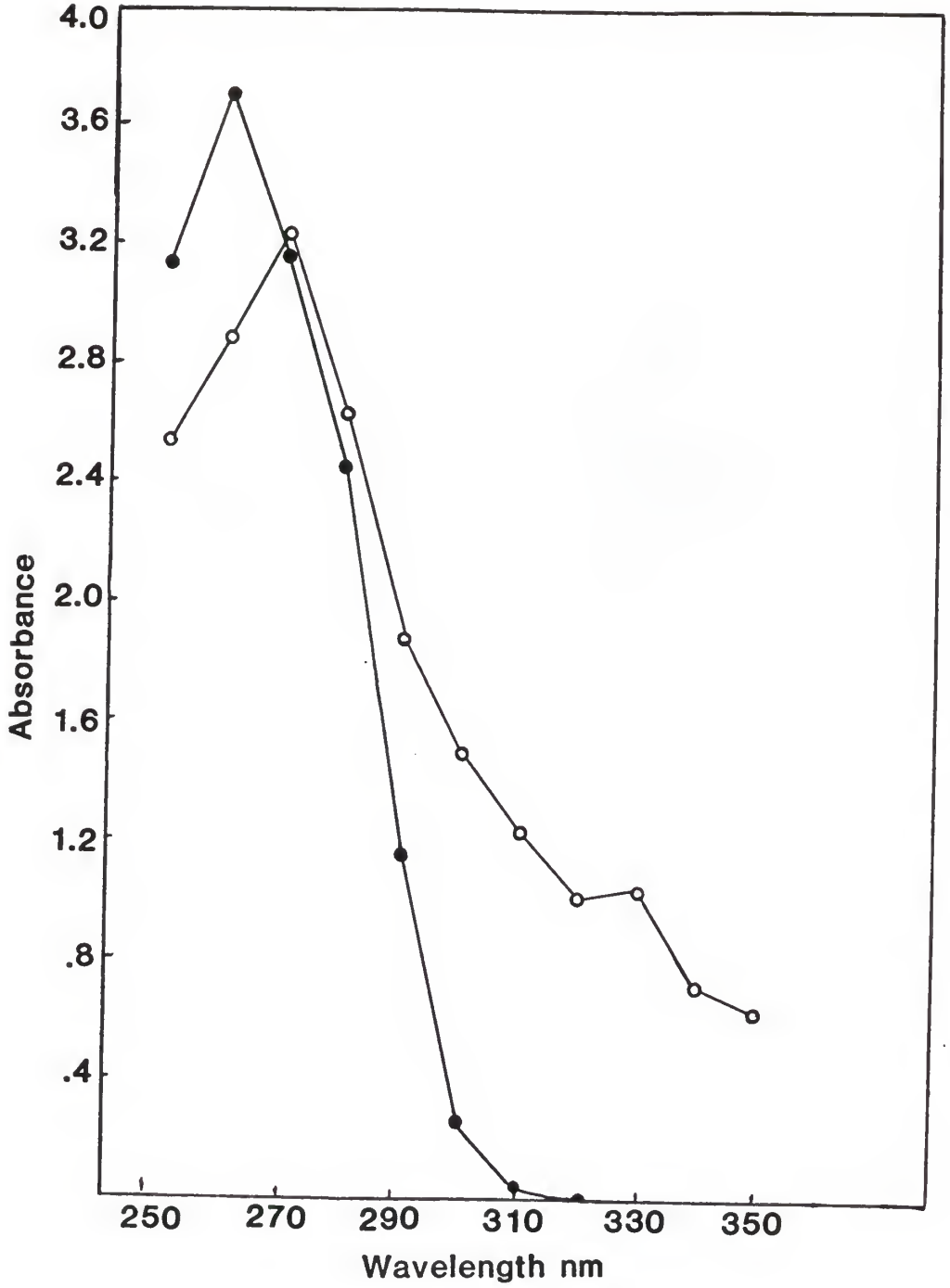

100
90
80
70
60
50
40
30
20
10
0
Percent Viability
4
8
12
16
20
Percent DMSO

Fig. 3. Absorbance Spectra of Sulfoxide 1 and DNA. The absorbance spectra of Sulfoxide 1, 0.1 mg/ml in 10% DMSO in PBS, (-o-), and calf thymus DNA, 0.5 mg/ml in 10% DMSO in PBS, (-●-), were measured between 250 - 350 nm.


4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
.8
.4
Absorbance
250
270
290
310
330
350
Wavelength nm

IV.

General Discussion

The search for new and improved drugs to better treat a variety of cancers has led scientists to screen plants, marine animals, microbial fermentation broths, and synthetic compounds. After ensuring that the active agent has a novel structure, agents from those sources which exhibit cytotoxicity in vitro are further screened for activity in vivo against a variety of tumors. The most promising compounds proceed through the screening process, until, finally, new drugs are licensed for clinical use (1).

It is not enough to know only that a drug is cytotoxic to tumor cells. The mechanism of action of a drug, as well as its demonstrated activities against a variety of tumors on the tumor panel, can help determine the most efficacious use of a drug in the treatment of cancer. In addition, possible side effects and dose-limiting toxicities are important to consider in designing chemotherapy programs. For example, the combination of both myelosuppressive and nonmyelosuppressive agents in multi-drug regimens helps to prevent the total annihilation of bone marrow progenitor cells while maintaining cytotoxic activity against the cancer (2).

The synthetic compound, 1-phenyl-3-phenylamino-4-(p-toluenesulfinyl)trans-1,5-hexadiene (Sulfoxide 1), was determined to be cytotoxic to P388 and L1210 murine leukemia cells in vitro. In order to determine the mechanism of this inhibition, synthesis inhibition studies using radiolabelled precursors were performed. Results indicated that both DNA and RNA synthesis were inhibited by Sulfoxide 1. Further work involving the time course of this inhibition suggested that both DNA and RNA synthesis were inhibited to

about the same degree after one hour of exposure to Sulfoxide 1. Because RNA is synthesized during the G1 phase of the cell cycle, and DNA during S phase, it would appear that Sulfoxide 1 is a phase and cycle nonspecific agent. Some of the alkylating agents, also phase and cycle nonspecific agents, are more active in a particular phase even though they are active in all phases (3). This may also be true of Sulfoxide 1. Further work using synchronized cells is indicated.

In order to determine the cytotoxicity of Sulfoxide 1 for non-cancerous cells, assays using normal murine splenocytes were performed. Cell viability, as measured by trypan blue dye exclusion, was determined for splenocytes which were stimulated or nonstimulated by the mitogen Con A. Cell viability of P388 tumor cells was determined for comparison. Results indicated that Sulfoxide 1 was preferentially cytotoxic to the tumor cells. Because the membrane composition of tumor cells is likely to be different from that of spleen cells, thus allowing differential uptake of trypan blue dye, another assay was performed using a different criterion of cell viability: the incorporation of tritiated thymidine. The results from this assay were quite different, as they indicated that Sulfoxide 1 was cytotoxic not only to tumor cells, but also to rapidly dividing (Con A- stimulated) spleen cells. This suggested that Sulfoxide 1 is toxic to proliferating cells, and also suggests that Sulfoxide 1 may have similar side effects as the other known anticancer drugs which are preferentially cytotoxic to proliferating cells: nausea, due to intestinal tract toxicity; alopecia (hair loss); and, myelosuppression (3). Again, further work is indicated to determine the side effects

of Sulfoxide 1. This is usually part of Phase I clinical trials, if the drug progresses to that point in screening.

After the cytotoxicity of Sulfoxide 1 for tumor cells in vitro was demonstrated, it was then necessary to determine its toxicity in vivo. However, due to the known toxicity of 100% DMSO to mice (4), attempts were made to limit the amount of DMSO injected by using 50% DMSO in buffered saline solutions. However, due to the nature of the interaction between DMSO and water, such that water preferentially hydrogen-bonds to DMSO (4), the solvating ability was lost, and Sulfoxide 1 precipitated out of solution. In addition, heat is generated upon mixing DMSO with water, and this may also have adversely affected the Sulfoxide 1. Suspensions of Sulfoxide 1 were injected, i.p., into mice, up to a calculated dose of 42.7 mg/kg body weight, with no significant deaths. Internal examination for gross pathological defects revealed liver necrosis as a major toxicity; however, again, other experiments are necessary to determine Sulfoxide 1 toxicity to bone marrow and other organs.

The in vivo activity of Sulfoxide 1 against implanted L1210 cells was then determined. Fifty percent suspensions of Sulfoxide 1 were used in a variety of injection schedules, with no success. It was possible that the tumor burden was already too great by the time of the first injection, or that the tumor had already metastasized. It was previously found that 2 to 4 days after implantation, P388 cells leave the peritoneal cavity (5). Alternatively, it is very likely that Sulfoxide 1, as a suspension, was not able to be taken up across the membrane into the tumor cell, thus, no cytotoxicity was observed.

An anomaly exists: a solution of Sulfoxide 1 in DMSO is added directly to aqueous media for in vitro assays, and no precipitation occurs, but cytotoxic activity is seen; a solution of Sulfoxide 1 in DMSO is added to aqueous solvent before being added to an assay, precipitation occurs, and no cytotoxic activity is seen.

In an attempt to increase the solubility/activity of Sulfoxide 1 in vivo, liposome encapsulation was tried. The method of liposome preparation seemed to be successful as long as sufficient quantities of lipid were present (to prevent the formation of a wax-like scum). Again, a variety of injection schedules of Sulfoxide 1/liposomes was tried, with no success. Perhaps an ineffective combination of lipids was used: lipid composition of liposomes has been shown to be important in drug delivery (6). Alternatively, the liposome may have been internalized by the tumor cell, but Sulfoxide 1 was trapped in the lysosome and was destroyed before it was able to exert its effect (7). During the preparation of the liposomes, it was necessary to heat to 60 ^{o}C for 1 minute. This temperature may have been deleterious to Sulfoxide 1, and may have helped to decompose the compound before it was injected. It is also possible that the ineffectiveness of Sulfoxide 1 in vivo is a result of the inherent differences between tissue culture systems and living animals. It is possible that Sulfoxide 1 is cleaved or inactivated by an enzyme, metabolized by the liver, or otherwise removed from the system before the compound has time to exert its effects. If this possibility is determined to be true, synthetic organic chemistry may be of value in preparing an analog which is not easily cleaved or inactivated, and

which shows activity in vivo. Finally, perhaps Sulfoxide 1 is not active against L1210 in vivo, but is active against other tumor systems. The illudins, for example, are not very effective against murine tumors, but show activity against human tumors (8). Further work on a variety of tumors is being conducted by the NCI.

It is not known how Sulfoxide 1 works. It is assumed that it must enter the cell to be effective; Sulfoxide 1 was not cytotoxic when dissolved in either ethanol or 50% DMSO in PBS. Dissolved in 100% DMSO, Sulfoxide 1 was very cytotoxic in vitro. As mentioned previously, it is not known why Sulfoxide 1 does not precipitate out when the DMSO solution mixes with the media in an in vitro assay. The insolubility of Sulfoxide 1 in water suggests that the compound is hydrophobic, and, as such, may be lipid soluble. Some of the compound's cytotoxicity may be due to membrane effects, as in the case of doxorubicin (9). Further work using radiolabelled Sulfoxide 1 may help determine whether the drug actually enters the cell, and how it is distributed within the cell.

It is thought that Sulfoxide 1 must be activated to be cytotoxic. The activation may occur in the following manner: In solution, the phenylamine of Sulfoxide 1 attracts an H^+ ion, which results in a positive charge on the nitrogen (see Figure 5). As aniline is a good leaving group, the positive charge is transferred to carbon-3. The aniline extracts the hydrogen from carbon-4, and a double bond between carbons 3 and 4 is formed, thus giving rise to a conjugated triene. This triene is then subject to attack by such nucleophiles as a guanine N^7 from DNA or RNA, or sulfhydryl from protein, thus giving

rise to an alkylation. Should another addition occur, crosslinks may be formed. The aniline itself may contribute to the cytotoxic effects of Sulfoxide 1. The question remains of how the triene reaches the DNA and RNA to exert these effects. One possibility is the following. It is known that DMSO is able to transport certain drugs as it moves. It is also known that DMSO passes through membranes by exchanging sites with bound water molecules. Inside the cell, proteins, nucleic acids, and polysaccharides are bonded together and covered by an "ice-like" sheath of water (10). As the DMSO comes into contact with these structures, the transported Sulfoxide 1 may then be able to exert its effects on the exposed portions of the proteins or nucleic acids. In this manner, the Sulfoxide 1 is protected from precipitation by the DMSO, and is delivered directly to the sites of action.

In conclusion, the synthetic compound Sulfoxide 1 is a cytotoxic compound which reacts in the same manner as an alkylating agent. Although cytotoxicity could only be demonstrated in vitro, with an improved solvent system or drug delivery system, Sulfoxide 1 could prove to be an effective new drug in the treatment of cancer.

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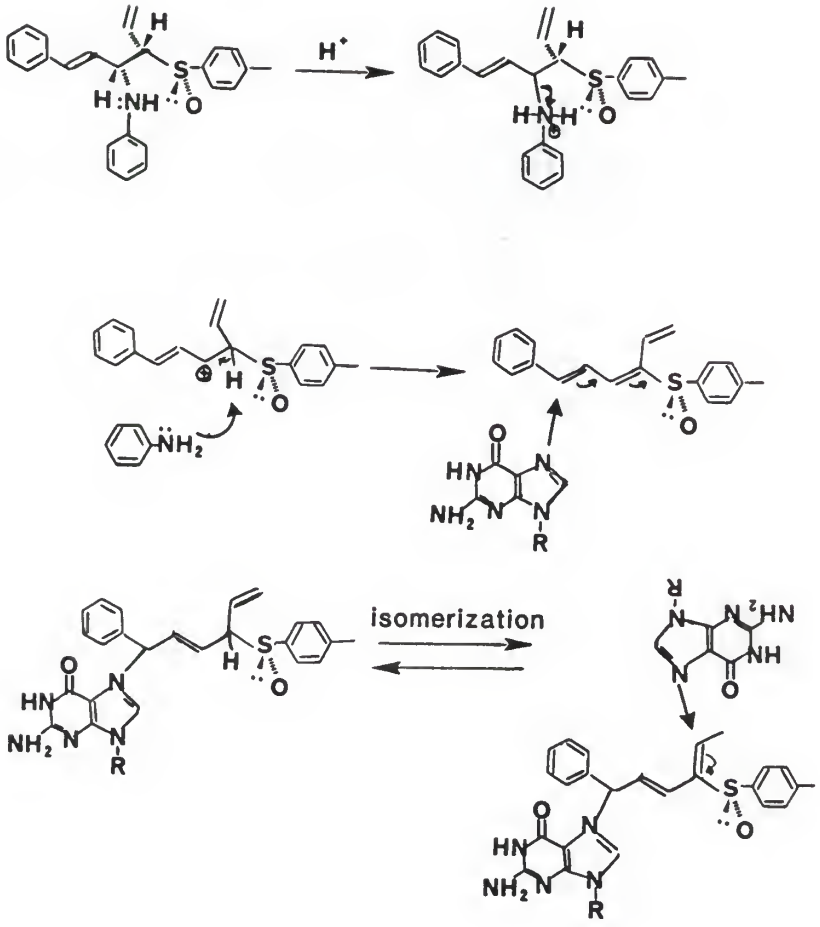
Fig. 1. Proposed Mechanism of Action of Sulfoxide 1. Sulfoxide 1 forms a triene in solution which is subject to nucleophilic attack by guanine N^7 of DNA or RNA. Mono- or bifunctional alkylations may occur.


H
H:NH
S
O
H+
isomerization
HN
NH2
N
R

STUDIES ON THE CYTOTOXIC EFFECTS AND MECHANISM OF ACTION OF 1-PHENYL-3-PHENYLAMINO-4-(P-TOLUENESULFINYL)-TRANS-1,5-HEXADIENE

by

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B.A., Gettysburg College, 1980

AN ABSTRACT OF A THESIS

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ABSTRACT

The synthetic compound, 1-phenyl-3-phenylamino-4-(p-toluenesulfinyl) trans-1,5-hexadiene (Sulfoxide 1), is an intermediate in the synthesis of an antibiotic carbapenum. Its novel structure is unlike other known anticancer agents. The compound exhibits in vitro cytotoxic activity against P388 (LD_{50}= 30 µg/ml) and L1210 (LD_{50}= 29 µg/ml) murine leukemia cells in culture. This cytotoxicity compares favorably with the activity of known anticancer drugs such as doxorubicin.

Cytotoxicity assays against normal mouse splenocytes suggest that Sulfoxide 1 is preferentially cytotoxic to proliferating cells. Both P388 tumor cells and mitogen-stimulated splenocytes showed greatly reduced cell viability after 24 hours of exposure to Sulfoxide 1. In contrast, normal mouse splenocytes were not significantly effected.

Toxicity studies in animal models suggest that Sulfoxide 1 is not overly toxic in vivo. The drug was not lethal at doses less than or equal to 42.7 mg/kg body weight when injected into mice, although liver necrosis was the major observable toxicity.

Studies of Sulfoxide 1 effectiveness against tumor-bearing mice suggest that Sulfoxide 1 is either inactive or inactivated in vivo, such that no cytotoxic activity is seen against implanted L1210 tumor cells. Neither drug suspensions nor liposome-encapsulation of the drug caused an increase in life span of treated mice over control mice.

Synthesis inhibition studies using radiolabelled precursors demonstrate that both DNA and RNA synthesis are inhibited by Sulfoxide 1 within one hour of exposure to the drug. This suggests that Sulfoxide 1 is a phase- and cycle-nonspecific agent, similar to the alkylating agents. There was no inhibition of protein synthesis following three hours of drug exposure.

Structure analysis suggests that Sulfoxide 1 is capable of reacting as either a mono- or bifunctional alkylating agent, similar to Mitomycin C. This mechanism of action is supported by the observed effects of this novel anticancer drug.